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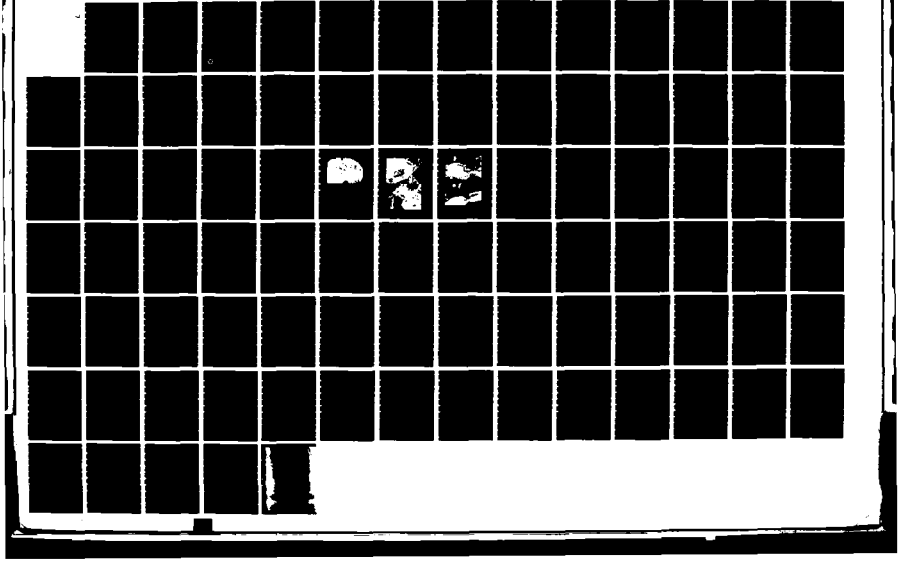

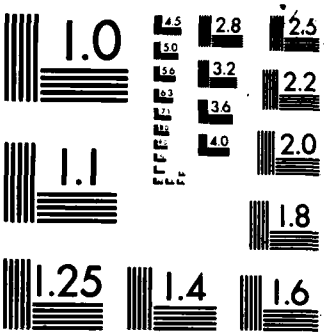

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ANNUAL TECHNICAL REPORT NUMBER 4

HORMONAL INTERFERENCE WITH PHEROMONE SYSTEMS IN PARASITIC ACARINES, ESPECIALLY IXODID TICKS

By

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The most important result of recent project research was the demonstration of the juvenoid JH III by radioimmunoassay. This assay revealed an estimated 78 pg/tick in the hemolymph of partially fed Hyalomma dromedarii females, and an estimated 3 pg/tick in the hemolymph of partially fed D. variabilis. Other studies, especially digestion of tritium labelled JH III, provided additional evidence suggesting the presence of this hormone in adult ticks. The implications of these findings for our understanding of sex pheromone regulation in ticks is discussed.

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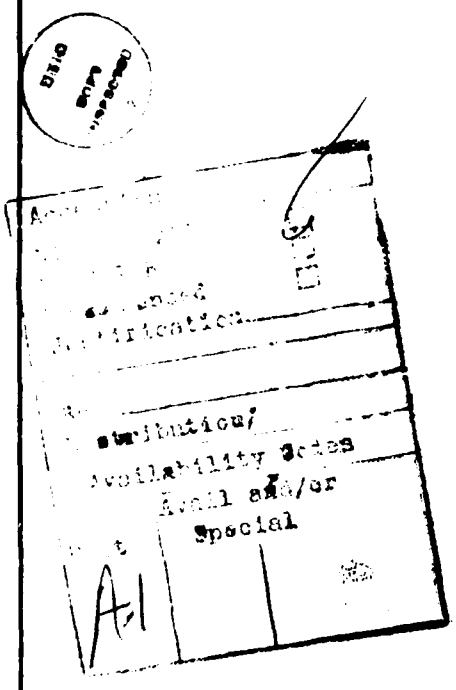
Other studies described in this report deal with the source of ecdysteroid in the camel tick, Hyalomma dromedarii, the American dog tick, Dermacentor variabilis, and the soft tick, Ornithodoros parkeri. Studies done at ODU, using radioimmunoassay high performance liquid chromatography, and autoradiography, provide new evidence implicating the tick synganglion - lateral nerve plexus as an important site of ecdysteroid activity in the ixodid ticks. Whether these findings reflect sites of ecdysteroid synthesis or merely storage is uncertain, although intense accumulations of ^{14}C labelled material in the synganglion (following inoculation of ^{14}C cholesterol) suggests the former. However, studies done at GSC with the soft tick, O. parkeri, found no evidence of molting hormone activity with synganglion transplants or other bioassays involving the synganglion of this species. (see below)

Other studies with ecdysteroids suggest the metabolism of ecdysone or 20-hydroxyecdysone (or both) to inactive metabolites, possibly including polar conjugates. If confirmed, these findings indicate the presence of only a single active ecdysteroid hormone in ticks, 20-hydroxyecdysone.

Studies with ecdysteroid analogues were continued, and confirmed the ability of such compounds to alter (accelerate) molting and excite sex pheromone activity in the camel tick, Hyalomma dromedarii.

Studies at GSC confirmed a regulatory relationship between 20-hydroxyecdysone and apolysis in the soft tick, Ornithodoros parkeri. New methods were developed to provide convenient and inexpensive but sensitive bioassays for ecdysteroid activity in this model species. One method which shows considerable promise uses parabiosis; other methods used ligation, synganglial transplants, and injection of synganglial extracts. All were done to determine whether ecdysteroids could be transferred from donor to recipient individuals and identify the organ(s) responsible for ecdysteroid production. The findings obtained by these innovative, experimental methods are compared with those of other workers, and their implications for our understanding of hormonal regulation of physiological processes in ticks are discussed.



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I. INTRODUCTION

This report describes continuing studies on the interactions of hormones and sex pheromones in ticks, as well as reproductive strategies in the Acari. Important new findings have been made in the course of this work, most notably, the discovery of ecdysteroid excitation of sex pheromone activity, and the widespread occurrence of the common molting hormone, 20-hydroexyecdysone (20-OH ecdysone). The latter is present in adult ticks, often in amounts exceeding that found in the immature stages, indicating its regulation of functions other than molting. The role of 20-OH ecdysone in vitellogenesis has been reported elsewhere (Diehl et al. 1982, review). Our findings indicate that regulation of sex pheromone activity is another equally important function of this hormone in ticks.

Having established the interaction of ecdysteroids and sex pheromone activity, our next concern is to determine the site(s) of synthesis and biosynthetic pathway(s) of the hormones. This report summarizes new findings indicating the synganglion and/or lateral organ system may serve as the site of synthesis (or storage) of these ecdysteroids in ticks. Other studies were directed to the use of cholesterol as a precursor of the biosynthetic pathway. Another problem concerns the presence of compounds which are radioimmunoassay positive, but nevertheless distinct from 20-OH ecdysone or ecdysone. Although the latter are the most common hormones of this class in insects, other active ecdysteroid hormones are known, e.g., Inokosterone and Makisterone. Acarines are arthropods, but they are quite distinct from insects and the possibility of hormones unique to this group can hardly be excluded. Compounds found in tick extracts, including several more polar than 20-OH ecdysone, bind to the highly specific antibody used in our radio-

immunoassay, implicating them as ecdysteroids. New evidence described in this report indicates that these compounds are steroids, and probably represent inactivation products of ecdysone metabolism.

The question of juvenile hormone occurrence in ticks has been the subject of intensive study by many investigators. Although impressive indirect evidence of JH activity has accumulated, direct evidence of specific JH compounds has not been reported. We report, for the first in Acari, specific and direct evidence of the juvenile hormone, JH 3, in 2 species of ticks as determined by a highly specific radioimmunoassay performed in France. If confirmed, this discovery is of major importance and will serve as the theoretical basis for all of our future studies of juvenoids in ticks. Other evidence of JH activity in ticks is described also.

II. JUVENILE HORMONE IN THE CAMEL TICK, HYALOMMA DROMEDARII: EVIDENCE FOR THE OCCURRENCE OF JH III IN FEEDING VIRGIN FEMALES.

Introduction

Although the occurrence of juvenile hormones is generally assumed (Solomon et al. 1982), no direct evidence of these molecules has been found in these acarines. Using the anti-allatotropin Precocene 2, Pound and Oliver (1979) demonstrated disruption of oogenesis in the soft tick, Ornithodoros parkeri. Treatment of the same individuals with exogenous JH resulted in limited oogenic activity and oviposition. These findings, first eliminating the hormone essential for oogenesis by means of chemical antagonism followed by "rescue" of the ovary exogenous JH, suggest the existence of a JH-like gonadotropic hormone. Other evidence of JH activity in ticks, both as a juvenilizing hormone and as a gonadotropin, is summarized by Solomon et al. (1982). Clearly, the accumulated evidence argues for the existence of JH in ticks. However, no direct chemical evidence of specific juvenile hormones in ticks has been reported. At present, identification of the specific tick juvenile hormone(s) is one of our most critical needs. The lack of such information limits further progress in our understanding of the hormonal regulation of tick developmental and reproductive processes, as well as studies to formulate anti-JH control measures, especially the disruption of mating by antagonism of hormones which control sex pheromone activity.

This study was undertaken to determine whether JH occurs in Hyalloma dromedarii and Dermacentor variabilis and, if present, to determine whether the tick JH resembles that of insects. Radioimmunoassay provided an ideal tool for preliminary investigations because of its high degree of specificity and extreme sensitivity, with detection possible with only a few pico-

grams. Experimental methods were used to determine JH esterase activity in tick hemolymph, a finding which would imply the presence of natural JH. Studies to develop chromatographic methods (HPLC, GC) were also undertaken to facilitate characterization of the type of JH found. Finally, bioassays were used to determine the sensitivity of H. dromedarii and D. variabilis to authentic insect JH applied to these ticks.

We report the first direct evidence of the existence of a specific juvenile hormone ever obtained in ticks, namely JH 3 or a very similar analogue. We also report evidence of JH esterase activity in tick hemolymph.

Materials and Methods

1. Collection and Extraction. Hemolymph was expressed from the severed joints of the legs of partially fed (7 days) virgin H. dromedarii and D. variabilis. The collections were kept cold (0-2°C) during their accumulation. A total of 385 ul was collected from H. dromedarii adults, and 700 ul from D. variabilis adults, respectively. The collections were diluted with 5 volumes of 1:1 methanol:diethyl ether. The samples were enclosed in flame sealed ampoules and forwarded to Prof. J. C. Baehr, Universite de Marie et Pierre Curie, Paris, France, for assay, using the methods described by Baehr et al. (1981). Samples of solvent were also forwarded to Prof. Baehr for use as controls. The samples were extracted in hexane, the hexane extracts purified by means of Silica Gel columns (Econo-Column, BioRad, Inc., Richmond, CA.) followed by further purification on C-18 Sep Paks (Waters, Inc. Milford, MA.). This procedure eliminated the most highly polar and the most highly non-polar components of the crude extract, without affecting the JH. The purified extracts were injected onto a Waters C-18

NovaPak 5 um column and chromatographed by means of a Waters, Inc. High Performance Liquid Chromatographic system. The retention times for insect JH 1, 2, and 3 were determined by injection of the authentic standards (Sigma Chemical Co., St. Louis, MO.). Fractions corresponding to the elution times were collected, concentrated, and tested by RIA. Further details of the extraction and purification procedure are given by Baehr et al. (1981).

2. Radioimmunoassay. This was done according to the technique of Baehr et al. (1981). The antiserum was prepared in rabbits using a JH conjugate (JH coupled with human serum albumin). Iodinated JH-Histamine (prepared by the chloramine-T technique) was used as the radiolabelled ligand. To perform the assay, phosphate buffer, 0.02 M, with BSA (1 part/100), pH 7.4, was used as the diluent. Samples of the extract were reconstituted in this buffer. Equivalent volumes (50 or 100 ul) of (1) sample, (2) iodinated ligand, and (3) diluted antisera were mixed in polystyrene tubes and incubated for 2 hours at 20°C. The reaction was stopped by the addition of dextran-coated charcoal. The precipitate was centrifuged and the radioactivity was counted on a gamma counter. Maximum sensitivity for the assay was estimated to be 20 picograms of authentic, homologous antigen. The anti-JH3 antiserum was considered to be the most specific of the several antisera produced.

3. JH Esterase Activity. Hemolymph was collected from part-fed virgin H. dromedarii females (fed 7 days) at 0°-4° C. A sample of 150 ul was inoculated with 1 uCi of ^{3}H JH3, 10 ul of a ethanolic solution containing 50 ug of non-labelled authentic JH3 (Sigma Chemical Co.) and 6 ml of 0.1 M phosphate buffer, pH 7.5. The mixture was allowed to incubate, with gentle shaking, for 30 min. at 30° C. The reaction was stopped by the addition of

250 ul of a mixture of methanol:water:ammonia, 10:9:1, and agitated vigorously for 1 min. Subsequently, 15 ml of iso-octane was added to partition the mixture into 2 phases. The extraction of the aqueous phase was repeated, and the iso-octane fractions combined. The organic phase was expected to contain the undigested JH, both labelled and unlabelled, while the aqueous phase was expected to contain the more polar diols and JH acids, if any, resulting from digestion of the parent molecules.

Controls were done and the results compared with the experimental observations described above. Two controls were used, namely, (1) as described above, but the hemolymph was inactivated by heating in a water bath for 60 min. at 80° C, and (2) as described above, but without hemolymph.

Analysis of the results of these reactions was done by radioassay and HPLC. Radioassay was done with a Beckman Liquid Scintillation Counter, Model LS 250, with an estimated efficiency of 42% for tritium beta rays. The scintillation cocktail was Dimilume (Packard Instrument Co., Downer's Grove, IL). HPLC was done with a Waters, Assoc. system (Milford, MA.), consisting of a Model UV detector (214 nm filter), 2 Model 6000 pumps, a u6K injector, a Z module for the column, Model 730 Data-Module, and a Model 721 Systems Controller. The column was a 10 um MicroBondapak C-18 column, 8 mm by 10 cm. The solvents were acetonitrile and water. Typical solvent ratios and pumping parameters were 75:25 and 1 ml/min., respectively. Under these conditions, JH1, 2 and 3 eluted at 12.42, 6.75, and 8.96 min. Sensitivity of the system, at 0.005 AUFS (maximum sensitivity) was Ca 2 ng.

4. Direct Chemical Analysis for Evidence of Juvenile Hormones. Studies to determine whether a direct chemical assay for juvenile hormones can be adapted for use with ticks are in progress. Two types of tick materials were used for extracts, namely (1) hemolymph, and (2) synganglion of part

fed females. Collections of 750 ul of hemolymph from replete D. variabilis females, 920 ul of hemolymph from virgin part-fed females, and 150 synganglia from part-fed females were used for these assay. Two types of analytical methods are being evaluated. In the first, after Connat (1982), the tick material is homogenized in cold acetonitrile (ACN) (with the aid of celite), filtered (fritted glass funnel), and the filtrate re-extracted several times with double distilled hexane:water (containing NACL), and the aqueous phase discarded. The organic phase is dried (NA_2SO_4), concentrated under nitrogen, and passed through a Waters Assoc., Silica Gel Sep Pak (Waters Assoc., Milford, MA.). The JH fraction is eluted with 100% acetonitrile. Further clean up is done by silica gel TLC (benzene:ethyl acetate, 94:6). The JH fractions are eluted (hexane:ethyl acetate, 90:10), dried, and reconstituted in ACN. The highly concentrated samples are assayed by HPLC, using a Waters Assoc. C_{18} column, 10 uM MicroBondapak, and a fixed wavelength UV detector at 214 nm, for evidence of juvenile hormones. The solvent system was cetonitrile and water, 75:25. The detector was set at 0.01 AUFS, peak width 5, and noise rejection 50. Sensitivity was estimated at ca. 1 - 2 ng with these conditions.

The second method under evaluation involves derivatization. Following formation of the derivatives, recovery and purification is done by thin layer chromatography (TLC) and High Performance Liquid Chromatography. These techniques were described in a recent paper by Hagenguth and Rembold (1979). In this procedure, the hormone is converted in a JH alcohol (by breaking the epoxide linkage) and esterifying it with Heptafluorobutyrlimidazole (HFB) to form the halogenated derivative. This derivative is readily detected with sensitives as low as 5 picograms using a gas chromatograph fitted with an electron capture detector (ECD). In view of the minute quan-

tities of JH likely to occur in ticks, this method is of great interest. Further details are given by the authors (Hagenguth and Rembold, 1979).

5. Biological Assays. Samples of authentic JH1, 2, and 3 were administered to D. variabilis nymphs to determine their effects on molting E and sex pheromone activity. Engorged nymphs, collected on the day of drop off, were inoculated with JH compounds dissolved in 2 ul of ethyl oleate, on the same day. D. variabilis nymphs were also treated while feeding, but the JH compounds were administered topically. The dorsal cuticle was abraded to facilitate penetration of the treatment, which consisted of 0.25 ug of each JH compound in 1 ul of dimethyl sulfoxide (DMSO). In addition to treatment with authentic insect JH, extracts of part-fed virgin female hemolymph (750 ul) were reconstituted in 200 ul ethyl oleate and administered to engorged nymphs by inoculation, 2 ul/nymph. Controls were done with solvents only (i.e., 2 ul ethyl oleate for inoculation, 1 ul DMSO for topical treatment). Ticks found dead or dying on the day of treatment were discarded. The remaining treated ticks were held in an Aminco Aire incubator at 80 ± 1° F and 90 ± 2% RH until they molted or died. The time of molting, if any, was recorded. Molted adults were examined for evidence of morphological abnormalities as well as alterations in normal sex pheromone activity. Table 2 summarizes the various treatments administered.

Results

1. Presence of JH3. No evidence of JH1, 2 or 3 was found in the samples assayed by HPLC, i.e., no peaks co-chromatographed with the same retention times as the authentic standards. When the fractions corresponding to these retention times were collected and assayed by specific RIA for each JH, evidence of immuno-reactivity was found only in the fraction cor-

responding to JH3. Consequently, a JH3 specific RIA was done on the remainder of the hemolymph collection. The results were as follows: 3.38 ng/ml of JH3 was found to be present in the hemolymph of part-fed virgin female H. dromedarii and 0.21 ng/ml in the hemolymph of part-fed virgin female D. variabilis. This represents 76.7 ± 3.1 pg/female H. dromedarii and 3.4 ± 1.3 pg/female D. variabilis.[1]

2. JH Esterase Activity. Table 1 summarizes the results of these studies. Radioassay of the 2 phases revealed 0.199 uCi of ^{3}H material in the aqueous phase, 0.098 uCi in the organic phase; 0.703 uCi was not recovered and presumed lost in insoluble precipitates. HPLC of the organic phase (done in accordance with methods described in section 3), revealed a single dominant peak at 8.95 min., the retention time of JH3. When co-injected with authentic JH3, the sample and the standard co-eluted. Thus, this fraction in the organic extract represents JH3. The fraction was collected and assayed for radioactivity; a 20 ul aliquot was found to have a count of 3678 CPM; this represents a total activity of 0.207 uCi of undigested JH3 remaining in the organic phase.

Radioassay of the controls revealed that very little ^{3}H activity remained in the aqueous phase after extraction. Before extraction with isooctane, 0.959 uCi was found in control No. 1, without hemolymph, and 0.808 uCi in control No. 2, with inactivated hemolymph (Table 1). Thus, almost all of the ^{3}H JH remained unaffected in control No. 1, while a substantial amount of the ^{3}H JH activity was lost just by binding to the macromolecules of the inactivated hemolymph in control No. 2. Following extraction of

[1] Based on an estimated hemolymph content of 22.7 ± 0.9 ul/part-fed female H. dromedarii and 15.9 ± 6.3 ul/part-fed female D. variabilis.

Table 1. Evidence of JH esterase activity in D. variabilis hemolymph.

Phase examined	Radioactivity (uCi remaining after extraction)		
	Active Hemolymph	Inactivated Hemolymph	No Hemolymph
Aqueous	0.199	0.015	0.076
Organic	0.098	0.221	0.791
Total	0.297	0.236	0.867

Table 2. Effects of treatment of D. variabilis nymphs with natural tick extracts and authentic insect juvenile hormones.

Treatment (administ.)	No. Treated	Molting time (days) $\bar{X}$ ± S.D.	% Molting
JH 1, 2.5 ug (Inoculation)	34	20.94 ± 0.23	50.0
JH 2, 1.0 ug (Inoculation)	40	——	0.0
JH 3, 5.0 ug (Inoculation)	28	19.0	14.3
JH 3, 2.5 ug (Inoculation)	40	19.4 ± 0.51	33.0
JH 3, 0.25 ug (Topical, 3 days prior to D.O.)	10	17.0	60.0
JH 3, 0.25 ug (Topical, 2 days prior to D.O.)	37	17.35 ± 2.10	92.0
Hemolymph extract (Inoculation)	103	16.6 ± 4.30	4.9
Controls, Ethyl oleate (Inoculation)	41	19.7 ± 0.03	30.0
Controls (Topical, solvent only)	77	18.1 ± 1.15	24.4

control No. 1 with iso-octane, 0.791 uCi was found in the organic phase, but only 0.076 uCi remained in the aqueous phase. Thus, only 7.9% of the ^{3}H JH in this control solution remained in the aqueous phase (the extraction was ca. 92% efficient). In constrast, extraction of control No. 2, inactivated hemolymph, revealed only 0.221 uCi in the organic phase, and 0.015 uCi in the aqueous phase. Again, only a small percentage of the ^{3}H JH activity remained in the aqueous phase, ca. 6.4%. However, the extraction was much less efficient than with control No. 1, only 27.4%; the rest was presumed lost as insoluble precipitates bound to the proteins of the inactivated hemolymph.

The resulted described above suggest that digestion of ^{3}H JH occurred in the presence of normal hemolymph, indicating the presence of JH esterases. Sixty seven percent of the tritium labelled material recovered was found in the aqueous phase of the experimental collection (active hemolymph) versus only 7.9% in the aqueous phase of control No. 1 (no hemolymph) and 6.4% in the aqueous phase of control No. 2 (inactivated hemolymph). JH esterase, if confirmed, would be consistent with our finding of JH 3 by radioimmunoassay.

3. Direct Chemical Assays. TLC of the D. variabilis hemolymph and synganglion extracts revealed a major spot resembling the retention time of JH III, although it did not co-chromatograph with it. Assay of this material from virgin, part-fed females by HPLC revealed a slight rise at the retention time for JH III, but no definite peak was recorded. No evidence of JH I or JH II was found. Assay of the material from replete females did not reveal any peaks that co-eluted with any of the juvenile hormone standards.

Studies with method 2, derivatization, are still in progress. Preliminary assays revealed multiple peaks indicating incomplete derivatization.

Studies directing at refining the technique are in progress, and will be completed prior to attempting derivatization of the tick extracts.

4. Biological Assays. Table 2 summarizes the results of studies with exogenous JH administered topically or by inoculation. Mortality due to the solvents was very high, from 70% to 75.6% in the controls. Of those controls surviving the treatments, the mean molting time was 18.1 to 19.7 days, which is very similar to the value obtained with other, untreated controls (Please see Section IV). There was no significant difference between these molting times. None of the treatment groups exhibited significant alteration of their molting time when compared with the controls.

Bioassays of the treated ticks did not reveal any excitation of the female sex pheromone system. Unfed females from treated nymphs were unattractive to sexually active males. Tests with partially-fed females to determine whether sex pheromone activity was suppressed are still in progress.

Discussion

The discovery of JH III in the hemolymph of D. variabilis and H. dromedarii adults by RIA represents the first direct evidence of this compound in ticks, presumably serving as a gonadotropic hormone. Although the similarity of ticks and other acarines to insects suggests similar endocrine regulation, specific compounds serving as juvenilizing agents or gonadotropic hormones remain to be identified in these animals. Evidence implicating the synganglion as the source of gonadotropic hormone in ticks is reviewed by Diehl et al. (1982) and Solomon et al. (1982). The results of studies with exogenous JH or JH mimics have led to ambiguous results. A recent study by Connat et al. (1983) demonstrated excitation of vitellogenesis and oviposi-

tion in Ornithodoros moubata following treatment with various juvenile hormones or their mimics. In this species, virgin females normally engorge, but few ever lay eggs. However, treatment with the test compounds induced a highly significant increase in the percentage of ovipositing females. Of special interest is the fact that JH I was more effective than isomeric mixtures of JH II or III or juvenile hormone mimics, and that farnesol and farnesol methyl ether were also effective. Once egg production was induced, the number of eggs laid was as least as high as that of normal, mated females of the same weight; i.e., excitation initiated an "all or none" response!

Most of the evidence of exogenous JH activity was obtained with argasid ticks. Few studies have been reported with ixodid ticks, and these results have been mixed. Except for the report of Hafez and Bassal (1980) on R. sanguineus embryos, other attempts to juvenilize ticks with exogenous JH or JH mimics were unsuccessful (reviewed by Solomon et al. 1982). Ioffe and Uspenskiy (1979) reported delayed molting in non-diapausing engorged Ixodes ricinus nymphs treated with Altozar, while Dees et al. (1982) noted derangement of feeding and oviposition in feeding, mated D. variabilis following treatment with Precocene-2. Considerable caution is required before accepting such results as evidence of JH or gonadotropic activity, since the effects of the treatments may represent generalized toxicity rather than selective disruption of endocrine mechanisms. More recently, Khalil et al. (1984) found limited evidence of delayed molting but not other forms of altered development in Hyalomma dromedarii treated with JH I. They concluded that the ticks did not respond to this compound other than by toxicity.

The demonstration of JH III, or an extremely similar molecule, by RIA, may clarify the endocrine picture in ticks. Clearly, confirmation of these

results is necessary, preferably by gas chromatography and mass spectrometric analysis of derivatives. However, the evidence of JH esterase activity in tick hemolymph is also supportive, while the HPLC studies, though inconclusive, also suggest JH III presence. Confirmation of JH III as the gonadotropic hormone and, by extension, the juvenile hormone, of ticks would represent an important contribution to knowledge of regulatory mechanisms in these parasites. Development of innovative pest and disease control measures is critically dependent upon a thorough understanding of the regulatory biology of the parasites to be controlled.

III. OCCURRENCE OF ECDYSTEROIDS IN SPECIFIC BODY ORGANS OF THE CAMEL TICK, HYALOMMA DROMEDARII, AND THE AMERICAN DOG TICK, DERMACENTOR VARIABILIS, WITH NOTES ON THEIR SYNTHESIS FROM CHOLESTEROL

Introduction

Although the presence of the steroid hormones ecdysone and 20-OH ecdysone in ticks is firmly established (Delbecque et al. 1978, Bouvier et al. 1982, Diehl et al. 1982, Germond et al. 1982, Dees et al. 1984a, b), little else is known about these extremely bioactive molecules. Recent studies by Germond et al. (1982) on the metabolism of ecdysteroids in Orinthodoros moubata indicated alteration of these hormones, suggesting an inherent capability for their biosynthesis and degradation in this species. Biosynthesis of the ecdysones may be assumed to begin with cholesterol, a sterol readily available in the tick's blood meal, in view of evidence of a similar pathway in insects (Riddiford and Truman, 1978).

In insects, ecdysone is synthesized in the prothoracic gland which serves as the major source of this hormone during the pre-adult life stages of these arthropods (Riddiford and Truman, 1978). Ecdysone is also secreted by the ovary in certain adult insects, e.g., the mosquito Aedes aegypti, and after conversion to 20-hydroxyecdysone, acts on the fat body to stimulate vitellogenin production, to be incorporated in the ova (Hagedorn et al. 1975, Hanoaka and Hagedorn, 1980).

No gland comparable to the insect prothoracic gland has been found in ticks and the source of the ecdysteroids, especially the more abundant 20-hydroxyecdysone, remains unknown. Binnington (1981) described evidence implicating an endocrine role for the lateral organs associated with the synganglion of the cattle tick, Boophilus microplus. However, no evidence

indicating a specific hormone was described. Synganglion structures believed to be comparable to the neuro-haemal organs of insect brain have also been reported (Obenchain and Oliver, 1974, 1975; Pound and Oliver, 1984) but their role as an endocrine center, if any, remains to be determined.

This study was undertaken to determine the distribution of ecdysteroids, predominantly 20-hydroxyecdysone, in the body organs and tissues of virgin adult females of the American dog tick, Dermacentor variabilis (Say) and the camel tick, Hyalomma dromedarii Koch. This information may provide a basis for determining the organ (or organs) responsible for ecdysteroid production in these parasites, as well as target organs and tissues affected by these hormones.

Materials and Methods

Ticks. The camel tick, H. dromedarii, was colonized and reared from specimens provided by the Medical Zoology Department, U. S. MANRU-3, Cairo, Egypt (HH#59723; U.S. APHIS permit #9433). The American dog tick, D. variabilis, was reared from specimens collected near Richmond, Virginia. H. dromedarii were fed in the laboratory on rabbits [Oryctolagus cuniculus]. D. variabilis immatures were fed on albino rats (Rattus norvegicus), while adults were fed on rabbits.

Tick organ and tissue samples. A total of 150 specimens of each species, H. dromedarii and D. variabilis, were used for these studies. Partially engorged females of each species, attached to rabbits for 7 days, were forcibly detached. Hemolymph was withdrawn from the specimens by severing one of the legs at the coxal-trochanteral joint and expressing the

fluid by gentle pressure. The exuded fluid was collected with Drummond Microcap micropipettes (Drummond Scientific Co., Broomall, PA.). Hemolymph contaminated with host blood was rejected. Collections were continued until 500 ul. (1st sample) or 1000 ul (2nd sample) were collected. The amount of hemolymph collected from each tick was measured using the Drummond microcap microliter micropipettes. Following the collection of the hemolymph, the ticks were dissected in cold (4°C) Shen's solution and the synganglion, foveal glands, salivary glands and ovary were removed from each specimen. The number of specimens used for these organ collections in each species is given in Table 3. In addition, the muscles and epidermis from the entire dorsal body cuticle were removed, and separate collection of these tissues were prepared. All organs or tissues were kept on wet ice (0°C) while the collections were accumulated.

The weight of each organ or tissue (except hemolymph) was determined by weighing individual specimens with a Cahn electrobalance (Ventron Inst. Co., Paramount, CA.) sensitive to ±0.1 ug. Individual specimens were washed in distilled water 3X, dried over Drierite (W.A. Hammond Drierite Co., Zenia, OH.) in a vacuum dessicator for 48 hrs at room temperature prior to weighing. Mean weights were determined for the organs of 20 specimens, except in the case of the foveal glands, where only 5 were used. To measure the weight of the muscle used for assay, a 5 mm^2 area of the dorsal integument, including the posterior third of the scutum was removed from 5 specimens of each species. The muscles were scraped from the cuticle, separated from tracheae, epidermis, fat body, and other tissues and dried as described above. The epidermis/fat body remaining was pipetted onto pre-weighed balance pans, dried, and weighed.

Table 3. Summary of estimates of total ecdysteroid content of selected body organs and tissues of partially fed virgin female Hyalomma dromedarii and Dermacentor variabilis

Organ/ Tissue	H. dromedarii No. in Sample	Amount ecdysteroid Mg. Tissue	pg/tick	pg/mg	D. variabilis No. in Sample	Amount ecdysteroid Mg. tissue	pg/tick	pg/mg
Syn-ganglion	250	5.16 ±1.31	2,479 ±1,134	120,223 ±24,361	250	5.14 ±1.11	1,901 ±1,308	92,416 ±20,406
Hemo-lymph	——	1.50	1,624* ±66	72	——	1.50	1,277** ±551	80 ±10
Sal. gland	25	26.93 ±8.55	312	290 ±92	25	22.45 ±2.88	957	1,065 ±120
Ovary	50	12.82 ±2.60	115	448 ±74	50	21.45 ±2.22	57	133 ±13
Muscle	25	9.58 ±4.13	98	226 ±46	25	4.42 ±1.63	190	1,074 ±289
Epidermis/ fat body/ Fov. gl.	10	12.59 ±0.91	500	397 ±27	10	4.87 ±0.24	1,120	2,300 ±122
Epidermis/ fat body	10	12.04 ±0.90	82	68 ±5	10	4.95 ±0.25	360	727 ±39
Foveal glands	49	0.67 ±0.33	158	11,731 ±6,977	50	0.39 ±0.07	133	17,183 ±823

* Avg./tick = 22.7 ± 0.9 ul.
** Avg./tick = 15.9 ± 6.9 ul.

Extraction. Extracts of the organs or tissue samples were prepared for radioimmunoassay and High Performance Liquid Chromatography (HPLC) as described by Dees et al. (1984 b).

Radioimmunoassay (RIA). Radioimmunoassay was used to estimate the amounts of ecdysteroids, as total ecdysteroid content, in the tick organ and tissue extracts. RIA was done as described by Dees et al. (1984 b). The Horn I-2 antiserum used in these assays was a gift from Dr. J. D. O'Connor. Standards included authentic ecdysone and 20-hydroxyecdysone (20-OH ecdysone) (Sigma Chemical Co., St. Louis, MO.). The labelled antigen, ^{3}H ecdysone (80 Ci/mmol) (New England Nuclear Corp., Boston, MA.) was adjusted by dilution to a count of ca. 4000 CPM. The standards and ^{3}H labelled antigen were checked for purity by TLC before use. Samples of each extract were assayed 3 times, unless noted otherwise, and 2 to 4 dilutions of each sample were assayed each time, depending upon ecdysteroid activity.

Chromatography (HPLC). The extracts described above were also assayed by HPLC for evidence of ecdysone, 20-OH ecdysone, or other ecdysteroids in the organ samples. HPLC was done as described by Dees et al. (1984 b). The system used (Waters Associates, Milord, MA.) included a model U6K injector, model 6000 pumps (2), model 721 systems controller, model 730 data module, and a model 441 dual channel UV detector using a 254 nm fixed wavelength filter. The columns used were reversed phase C-18 Novapak columns, containing 5 um C-18 packing. The solvents used were methanol (Burdick and Jackson Laboratories, Inc., Muskegan, Michigan) and Milli-Q filtered water (Millipore Corp., Bedford, MA.). Typical solvent ratios were 45:55, methanol: water at 1 ml/min. Standards included ecdysone, 20-OH ecdysone, Makisterone A (Sigma Chemical Co.) and Inokosterone (from M. Thompson, USDA, Beltsville,

MD.). The extracts were dried (N_2) and reconstituted in 50 or 100 ul of HPLC grade methanol. To enhance detection of the ecdysteroids, the detector was operated at or near its maximum sensitivity (0.005 AUFS).

Precursor(s) of Ecdysteroid Biosynthesis. To determine whether cholesterol was utilized in the incorporation of ecdysone or 20-OH ecdysone, H. dromedarii nymphs were inoculated on the day of drop off with ^{14}C labelled cholesterol (60 mCi/mmol, New England Nuclear Corp., Boston, MA.). The ^{14}C cholesterol was dissolved in a mixture of ethyl oleate and olive oil, 1:1, v/v. Aliquots of 3 ul contianing ca. 0.50 uCi was administered with a Hamilton syringe and 30 gauge needle. Assay of a sample of 10 inoculated nymphs revealed an average of 0.427 ± 0.104 uCi/nymph. Following treatment, samples of 10 surviving nymphs were sacrificed on days 2, 4, 8, 12, and molting day, and extractions were prepared in the same manner as described above. Others from the treated population were allowed to mature for 2 weeks after molting whereupon 10 males and 10 females were sacrificed and extracts of these samples were also prepared. The extracts were concentrated, aliquots were injected into the HPLC, and all peaks, including the 20-OH ecdysone peak and the location where ecdysone would have emerged if present, were collected. Authentic ecdysone was introduced into the samples to facilitate collection of any of the natural hormone that might have been present in amounts too small to be detected. The entire extract was used in each case and all of the peaks were accumulated. The sample peaks were concentrated [N_2], checked for purity (reinjected and separated further if impure), dissolved in the scintillant Dimilume (Packard Inst. Co., Oak Grove, IL.) and assayed for radioactivity with a Beckman Liquid Scintillation counter LS250 (Beckman Inst. Co., Fullerton, CA.).

Autoradiography. Engorged H. dromedarii nymphs were inoculated as described above, and samples of 2 or 3 individuals were collected at the time periods described previously. Two weeks after molting, samples of synganglion, foveal glands, salivary glands, ovary or testis, and integument were removed from the labelled males and females, quick frozen with Cryokwick (IEC, Nedham Heights, MA), embedded with Tissue Tek II O.C.T. embedding medium (Lab-Tek Products, Naperville, IL) and sectioned with a Harris CTD Cryostat (IEC). The frozen sections were mounted on slides, and dipped in liquefied nuclear emulsion NTB-2 (Kodak, Rochester, NY) in a dark-room. The specimens were dried, sealed, and exposed to the incorporated radioactivity for 2 weeks at 4°C. Subsequently, the slides were developed in 1:1 Dektol solutin (Kodak), fixed, stained with Delafield's hematoxylin and eosin Y, and examined for evidence of silver grain accumulation with a Nikon Model HFM 35 mm camera attached to the microscope. Samples of nymphs were collected at the times noted above and examined using the same techniques, but without dissection.

Results

Presence of Ecdysteroids in Tick Tissues. RIA demonstrated the presence of substantial amounts of ecdysteroids in selected tick body organs or tissues (Table 1). The highest concentrations of ecdysteroids was found associated with the synganglion, with ca. 1.9 ng/tick in D. variabilis females and ca. 2.5 ng/tick in H. dromedarii females. When considered on the basis of the mass of tissue represented by this organ (i.e. in pg/mg tissue), it is apparent that relatively large amounts of ecdysteroids are present in these vary small (ca. 20 ug) organs. The next largest accumulation

occurred in the hemolymph, in excess of 1 ng/tick. Of special interest is the presence of ecdysteroid accumulation in the foveal glands. When considered on the basis of their tissue mass (from 7.6 to 15.0 ug), the amounts of ecdysteroids which appear to be accumulated in or around these tiny glands represent the second highest concentration in any of the tick body organs. The samples of epidermis and fat body tissues which contained the foveal glands also had significantly more ecdysteroid than those without foveal glands, especially in the case of D. variabilis ($T = 36.8$, 18 d.g., p 0.001). This finding appears to be consistent with the hypothesis that ecdysteroid actually occurs in the foveal gland system, and not merely in fat body or epidermis adhering to the excised tissue samples. Exdysteroid was also found associated to some degree with all of the other organs or tissues examined. In H. dromedarii, the estimated amounts of ecdysteroid per tick for salivary glands, ovary and muscle were relatively small (from 98 pg/tick to 312 pg/tick), and were especially small when considered in terms of the mass of tissue represented by these structures. The cumulative total for these 3 organs and tissues, 525 pg/tick, represents only 11.1% of the total estimated ecdysteroid content of all the organs and tissues assayed, even though these 3 organs and tissues average 2.07 ng/tick. In D. variabilis, the cumulative total for these same 3 organs and tissues was 1025 pg/tick, representing 21.9% of the total estimated content of all body organs and tissues assayed.

HPLC demonstrated the presence of 20-OH ecdysone in the synganglion and hemolymph extracts of both H. dromedarii and D. variabilis. The amounts of 20-OH ecdysone found in H. dromedarii synganglion and hemolymph extracts, respectively, were as follows: 2.9 ± 0.2 ng/tick and 10.4 ng/tick (one

sample); in the D. variabilis synganglion and hemolymph extracts, respectively, 3.73 ± 0.7 ng/tick and 11.3 ± 5.5 ng/tick. No evidence of 20-OH ecdysone was found in any of the other organs or tissues. No evidence of ecdysone, Makisterone or Inokosterone was found in any of the extract samples assayed.

Autoradiography. Autoradiography of frozen sections of adult synganglia (from ^{14}C cholesterol inoculated nymphs) demonstrated evidence of substantial incorporation of ^{14}C into these tissues (Fig. 1-5). No evidence was found between males and females, or between mature unfed adults and part fed adults (fed 3 days). Much higher accumulations of silver grains, too numerous to count, occurred over the cortical zone and the lateral nerve plexus than over the medullary zone of the synganglion. At higher magnifications, e.g., 400 X, it was possible to observe clusters of silver grain accumulations over discrete areas (nerve centers?), from 31 to 82 grains/100 um^2 (n=3) (X=45 grains/100 um^2) near the edge of the cortex and in the lateral plexus (Fig. 2, 3). No other tissue or organ revealed intensities of silver grain accumulations comparable to that found with the synganglion and its adjacent nerve plexi. However, significant accumulations were found over the ductular zone of the foveal glands (from 28-96 grains/100 um^2 (n=4) (X=49 grains/100 um^2) (Fig. 4), as well as the epidermis and ducts of the dermal glands (Fig. 5). Significant accumulations were also found in one specimen over samples of the testis (37 grains/100 um^2). Background ranged from 9 to 15 grains/100 um^2 (n=5).

No evidence of significant silver grain incorporation was found in tissues of ^{14}C cholesterol injected H. dromedarii nymphs, or in unfed adults on the day of emergence. Silver grains were dispersed in a diffuse manner throughout the body, without specific accumulations.

Fig. 1

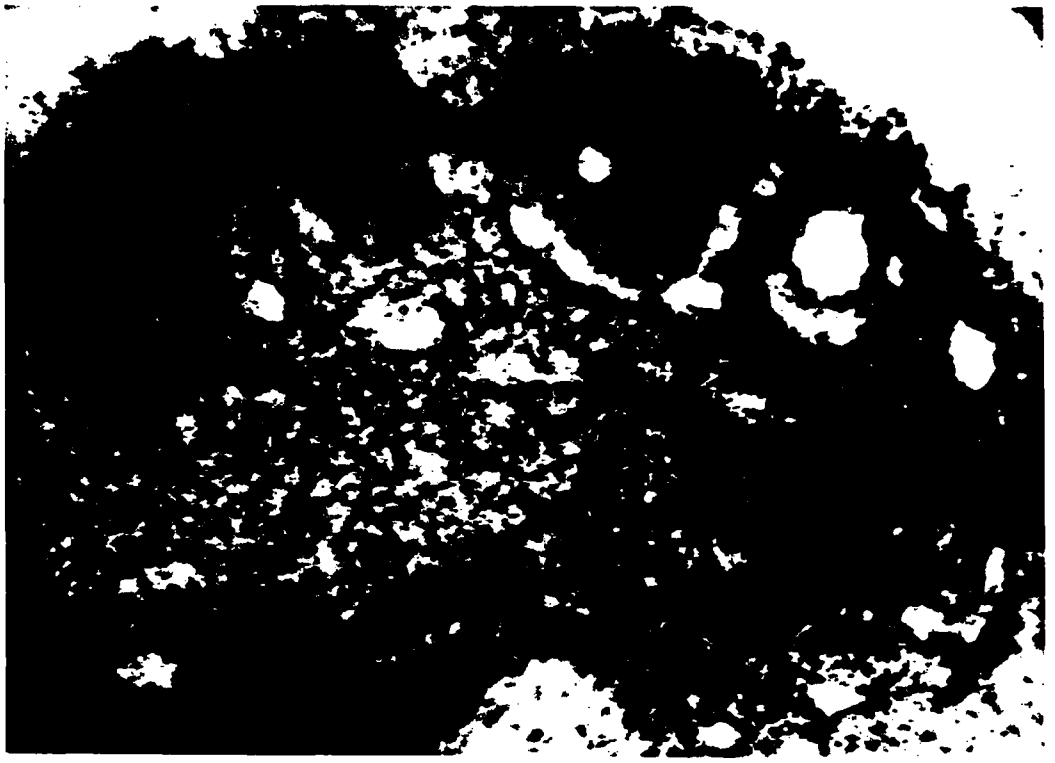


Figure 1. Autoradiograph illustrating dense silver grain accumulation reflecting ^{14}C activity in the synganglion and lateral nerve plexus of a part-fed female virgin H. dromedarii inoculated with ^{14}C cholesterol following nymphal engorgement. Most of the activity is centered in the cortical zone and lateral nerve plexus. 100X

Figure 2-3. Enlargements of the lateral nerve plexus illustrating highly significant silver grain accumulations over tissues (lateral organs?) between major nerves in the lateral nerve plexus. 400X

Figure 4. (Top) Autoradiograph illustrating scattered clusters of silver grains over the ducts and pore tubes of one of the paired foveae dorsales. 200X

Figure 5. (Bottom) Autoradiograph illustrating significant accumulations of silver grains near the foveal gland lobes and associated ducts. 100X

^{14}C labelled ecdysteroids. Radioassay of the 20-OH ecdysone fraction collected by HPLC revealed highly significant radioactivity, 893 CPM and 901 CPM (0.000437 and 0.000441 uCi) for the samples from female and male ticks, respectively. When the tests were repeated with another group of ^{14}C-cholesterol inoculated nymphs and the 20-OH ecdysone fractions collected from extracts of 10 females and 10 males, 760 CPM was found in the 20-OH ecdysone from the females, 180 CPM in the 20-OH ecdysone from the males. When the same procedures were used with the 20-OH ecdysone fractions from ^{14}C-cholesterol treated nymphs prior to molting, the results were very different. No ^{14}C activity was found in nymphs collected 4 and 8 days post-inoculation, and only 38 CPM in nymphs collected 12 days post-inoculation.

Radioactivity was also found in 3 large peaks more polar than 20-OH ecdysone. In the fractions collected from the females, the amounts of ^{14}C activity of these peaks, in order of decreasing polarity, were 45, 71 and 68 CPM, repectively; from the males, 15, 23, and 18 CPM, respectively.

Although evidence of ^{14}C incorporation was found in brain and epidermal samples from 30 females (356 and 264 CPM, respectively) and from 29 males (162 and 141 CPM, respectively), no radioactivity was found in the fraction where 20-OH ecdysone was expected to elute.

Discussion

These results implicate the synganglion and adjacent nerve plexi as the major center for ecdysteroid accumulation in the feeding virgin female. This nerve mass contained far more ecdysteroid than any other body organ or tissue. Hemolymph ranked second, after the symganglion and its surrounding plexi, in estimated total accumulated ecdysteroid activity/tick. The latter

represents a very conservative estimate. It is probable that the true amount of ecdysteroid in the hemolymph in these ticks was considerably larger than these estimates indicate, since it is doubtful that all of the hemolymph present was expressed from these ticks (the amount per milligram, however, is unaffected by this factor). The presence of ecdysteroid associated with the remaining organ or tissue samples is not unexpected. Hemolymph coagulates rapidly around these structures during their excision just as it does when allowed to spread over the external surface. However, in contrast to its ready removal from the waxy cuticle, it is virtually impossible to dislodge coagulated hemolymph from the internal structures despite repeated washing. Consequently, the occurrence of ecdysteroid activity associated with such tissues as muscle or salivary gland probably reflects unavoidable contamination with hemolymph rather real occurrence of ecdysteroids. This interpretation does not appear to apply to the epidermis and fat body, or the foveal gland (see below).

The finding of abundant accumulations of ecdysteroid activity in the synganglion and lateral plexus supports Binnington's (1981) hypothesis that the lateral segmental organs are endocrine organs probably engaged in the secretion of ecdysteroids (Binnington also allowed for the possibility that these organs may secrete JH, rather than ecdysteroids, or other, unknown substances). Although the only ecdysteroid in these structures that we could confirm by chemical assay (HPLC) was 20-OH ecdysone, ecdysone (its precursor) is almost certainly present, even if only in very small amounts. Diehl et al. (1982) noted that the amounts of ecdysone they found in specimens of A. hebraeum they examined were only 5% of the amounts of 20-OH ecdysone present in these same animals. Dees et al. (1984) were unable to

detect ecdysone in whole tick extracts of H. dromedarii, even when 20-OH ecdysone was very abundant.

The finding of ^{14}C incorporation into 20-OH ecdysone provides additional evidence of the biosynthesis of ecdysteroids by ticks. The synthesis of this class of steroids from cholesterol has been reported in several species of insects (Riddiford and Truman, 1978). Conversion of ecdysone to 20-OH ecdysone was demonstrated to occur in ticks by Bouvier et al. (1982), as well as in insects.

If cholesterol is indeed the precursor (or, at least one of the precursors) of ecdysteroid biosynthesis in ticks, the finding of substantial concentration of ^{14}C activity in the synganglion, the pheromone glands, and the epidermis takes on added significance. Although the ^{14}C activity found in the integument probably reflects cholesterol excretion (Cherry, 1969), that found in the synganglion and foveal glands may also include ^{14}C labelled ecdysteroids as well as other steroids, intermediates, and cholesterol.

IV. ECDYSTEROIDS IN TICKS: RADIOIMMUNOASSAY POSITIVE COMPOUNDS OTHER THAN 20-HYDROXYECDYSONE: CONJUGATES OR METABOLITES

Introduction

In addition to 20-hydroxyecdysone (20-OH ecdysone), the ubiquitous ecdysteroid of arthropods, at least 2 other fractions separated by High Performance Liquid Chromatography (HPLC) were found to react to the Horn I-1 radioimmunoassay. The identity of these steroids, or putative ecdysteroids, is unknown. In insects, conjugates of ecdysone and glycosides or other compounds occur (Koolman et al. 1973; Isaac et al. 1982) providing a means of storing the hormone for later use. Consequently, one possible explanation of the radioimmunoassay (RIA) positive activity of these other compounds found in ticks is that they too are conjugates, with sufficient parts of the steroid exposed to allow binding with the antigen. Another explanation suggests that they are by-products (inactive) or ecdysteroid metabolism.

This study was done to determine (1) whether enzymatic hydrolysis with glucuronidases and sulfatase would increase the amount of ecdysteroid detectable by RIA, and (2) whether the unknown RIA positive compounds also steroids.

Materials and Methods

A tick extract was prepared as described by Dees et al. (1984 a), using partially fed Dermacentor variabilis nymphs. The nymphs were processed when they had incubated for 10 days after engorgement. A total of 11,963 nymphs were used to prepare the extract. The extract was stored in 100% ethanol,

and aliquots were assayed or separated further for use in the various studies described below.

To separate the free ecdysteroid(s), especially 20-OH ecdysone, from the putative conjugates and other polar compounds with low RIA activity, 100 ul aliquots of the crude extract were dried, reconstituted in 10% methanol, and loaded onto pre-conditioned C_{18} Sep Paks (Waters Associates, Milford, MA.). The contents were eluted sequentially with solutions of increasing strength HPLC grade methanol (Burdick and Jackson, Muskeegan, MI.) and Milli-Q water (Millipore Corp., Bedford, MA.). To ensure that all compounds of interest remained on the C-(18) packing during loading, 2 Sep Paks were aligned in tandem, and the initial eluate was collected and returned to the column. This step was repeated 2 X. Subsequently, three elutions were carried out using (1) 10% methanol, (2) 30% methanol, and finally, (3) 100% methanol. In this elution scheme, 10% methanol was not expected to elute any steroids or conjugates; 30% methanol was expected to elute only moderately polar conjugates and, perhaps, some of the more polar steroids, if any exist; 100% methanol was expected to elute all remaining compounds, including ecdysone and 20-OH ecdysone. The eluates were identified as (1) Fraction 1 (10%), (2) Fraction 2 (30%), and (3) Fraction 3 (100%). These fractions were either assayed by RIA or subjected to enzymatic hydrolysis before further analysis was done.

Enzymatic hydrolysis was carried out with a mixture of Beta-glucuronidase, Types H-1 and L-II, also containing sulfatase activity, and Beta-glucuronidase Type 1 (no sulfatase activity) (Sigma Chemical Company, St. Louis, MO.). The enzymes were dissolved in 3 ml of a 0.2 M NACL solution, containing 7.4, 7.4, and 4 mg, respectively, of each of the above mentioned

enzymes. An aliquot of the tick extract was added to the solution, followed by a buffer (0.2 M solution of sodium acetate:acetic acid, 7:3, v/v) to bring the solution to a pH of 5; the final volume was 12 ml. The enzyme-extract mixture was incubated at room temperature for 24 hours. Enzyme action was terminated by the addition of 1 ml of a mixture of methanol: butanol, 1:3 (v/v), dried slowly (under N_2), and reconstituted in 100% methanol. Aliquots of the post-hydrolysis extract were assayed by RIA; the balance was loaded on C_{18} and separated into fractions (as described above) to determine whether additional free ecdysteroid was obtained as a result of the enzyme treatment.

HPLC was used to determine whether the C_{18} Sep Pak separation was eluting the ecdysteroids in accordance with the scheme described above. HPLC was done with a Waters Associate (Milford, MA.), Model 721 Systems Controller, Type 730 DataModule, Model U6K injector, Model 441 UV absorbance detector equipped with a 254 nm UV detector, and Z-Module radial compression to house the column. The column was a 5 uM NovaPak C-18 cartridge, 8 mm I.D. by 10 cm long. The solvent system was methanol:water (typical solvent ratios were 55:45, v/v, and the flow rate was 1.0 ml/min). Known amounts of authentic 20-OH ecydsone were loaded onto the C_{18} Sep Paks and eluted as described above. In addition, known of authentic 20-OH ecdysone were added (spike) to the crude extract and the mixture dried, reconstituted, and eluted with the C_{18} Sep Pak as described above. After elution and concentration, samples of each eluate were injected into the HPLC and assayed for evidence of ecdysteroids.

HPLC was also used to profile the crude extracts and collect fractions previously reported to react in the RIA, including 20-OH ecdysone, as well

as other fractions that may be of interest. A total of 8 fractions, including 20-OH ecdysone, were collected from the crude extract. The absolute amounts of material in each fraction were determined weighing aliquots of the entire combined collection of that fraction with a microbalance (Cahn Electrobalance, Ventron Instruments, Paramount, CA.). Each fraction was subjected to enzymatic hydrolysis as described above. The reaction mixture was agitated on a Vortex Mixer (Scientific Instruments, Inc., Queens Village, N.Y.), centrifuged, extracted and reconstituted as described above. Aliquots of the post-hydrolysis extracts of each fraction were assayed again for ecdysteroid activity by RIA and HPLC.

Chemical classification. To determine the identity of the RIA positive (presumed) steroids, aliquots of the fractions collected by reverse HPLC as described above were sent to the Magnetic Resonance Laboratory, University of South Carolina, Columbia, S.C., for analysis. The instrument used was a Bruker WH-400 Fourier Transform Nuclear Magnetic Resonance (NMR) system. Proton NMR spectra were obtained in mixtures of deuterated methanol and deuterated acetone. Proton NMR spectra were obtained for 3 highly purified fractions corresponding to (1) a highly polar compound, fraction 1, (2) a highly polar compound, fraction 2, and (3) a moderately polar compound, fraction 3, (4) authentic ecdysone, as a standard, and (5) authentic 20-OH ecdysone, also as a standard.

Mass spectroscopy was done with a Hewlett Packard Model 5992 GC-Mass Spectrometer with a direct insertion probe. Ions were bombarded with a 70 electron volt beam. Typical oven conditions were ca. 92°C. Spectral scans were collected over a range of ion fragments, from 55 to 600 mass units. Aliquots of the same fractions collected and purified by reverse phase HPLC

as described above were dried, reconstituted in 100% methanol to concentrations of ca. 50- 100 ug, and deposited in the solid probe container. In addition to these unknown compounds, authentic ecdysone, 20-OH ecdysone, and highly purified cholesterol (Sigma Chemical Co., St. Louis, MO) were used as standards.

Infrared spectroscopy was done with a Nicolet Model 5000 (Minneapolis, MN) instrument, at the Department of Chemical Sciences, Old Dominion University. The same compounds collected and purified by reverse phase HPLC as described above were ground in KBR. At least 0.5 mg of each fraction was estimated to be available for each compound assayed. In addition, 0.1 mg of authentic ecdysone and 20-OH ecdysone were also ground in KBR and spectra of these standards were obtained for comparison.

Results

Effects of enzyme hydrolysis on ecdysteroid content. RIA of the original crude extract prior to enzymatic hydrolysis revealed an estimated 1.3 ng/tick. Following enzymatic hydrolysis, an estimated 2.0 ng/tick was estimated by RIA, an increase of only 1.6 times the original amount.

The results of RIA of the fractions obtained by sequential elution of the extract on C_{18} Sep Paks are summarized in Table 4. Prior to enzymatic hydrolysis, only 1.6 and 2.7 ng RIA positive activity was found in fractions 1 and 2, respectively, representing compounds or conjugates more polar than 20-OH ecdysone; almost all of the ecdysteroid activity, 96.5% remained in fraction 3, which was eluted with 100% methanol. Following enzymatic hydrolysis, the amounts of RIA positive material in fractions 1 and 2 increased approximately 2 X, but the amount in fraction 3, where the 20-OH

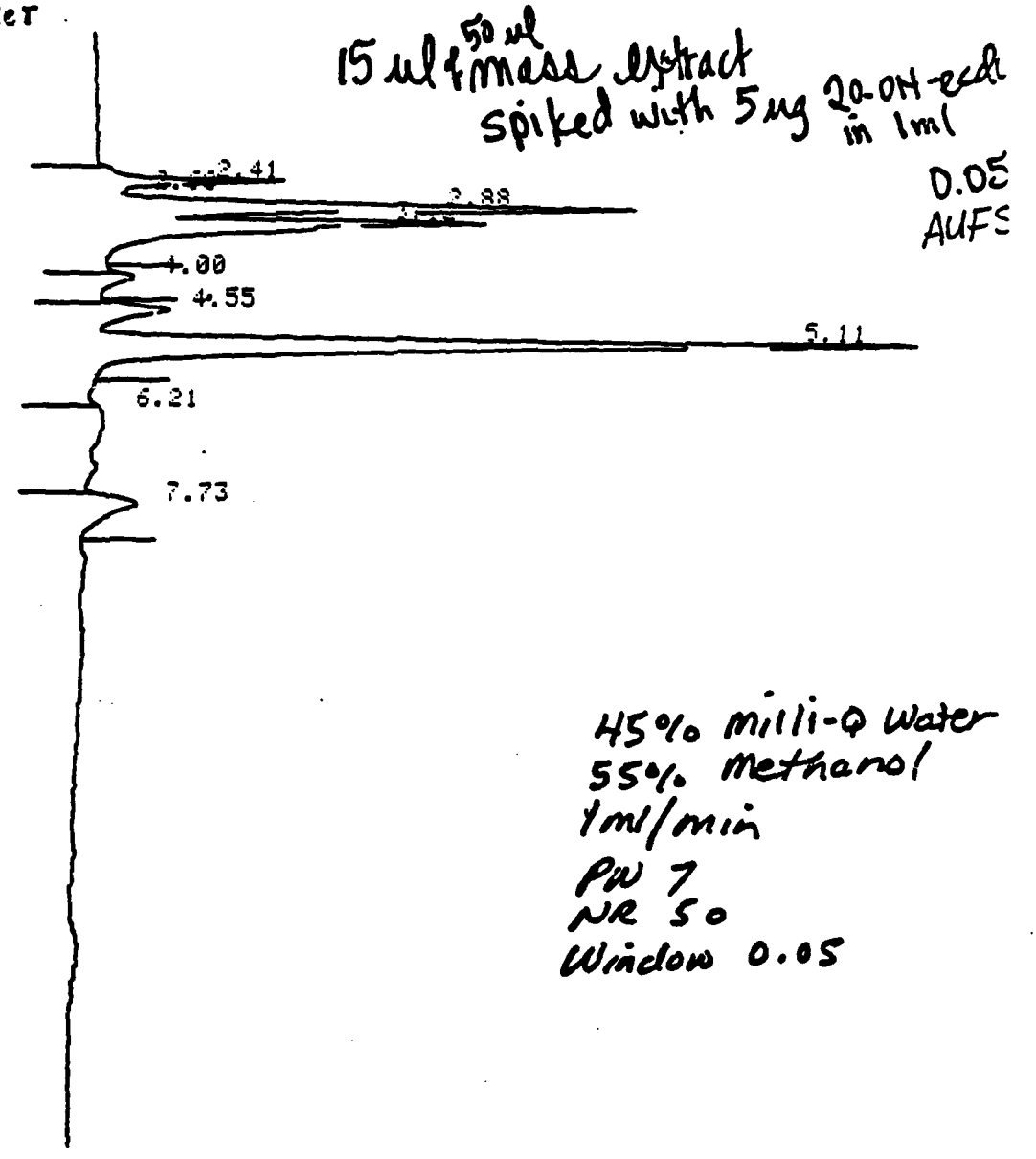
Table 4. Radioimmunoassay of eluates of a D. variabilis crude extract fractionated with a C-18 cartridge (Sep Pak) and methanol elutions.

Fraction	Prior to hydrolysis ng ecdysteroid (% of total)	After hydrolysis ng ecdysteroid (% of total)
10% methanol	1.6 (1.3%)	3.5 (2.8%)
30% methanol	2.8 (2.2%)	6.1 (4.8%)
100% methanol	122.1 (96.5%)	116.9 (92.4%)

ecdysone was eluted, remained unchanged. Overall, the proportions of RIA positive polar steroids vs. 20-OH ecdysone remained unaffected by the enzyme treatment. If the polar compounds in fractions 1 and 2 were polar conjugates of ecdysone or 20-OH ecdysone, the amount of RIA ecdysteroid activity in fraction 3 should have increased greatly, while that in fractions 1 and 2 should have diminished or disappeared.

The results of HPLC analysis of the C_{18} Sep Pak sequential elution of the crude extract are summarized in Figures 6-9. HPLC demonstrated that all of the 20-OH ecdysone was eluted in fraction 3, 100% methanol elution, during fractionation with these cartridges. Figure 6 is a chromatogram of an aliquot of the crude extract prior to separation on the Sep Paks; major peaks at 2.88 and 3.12 minutes post-injection are RIA positive; Figure 7 shows the polar compounds eluted in fraction 1, 10% methanol; Figure 8 shows those eluted with 30% methanol; Figure 9 shows those eluted with 100% methanol, after removal of the previous compounds. All of the 20-OH ecdysone is eluted in fraction 3, i.e., after elution of the more polar steroids, and only when the cartridges are flushed with 100% methanol. The amount of 20-OH ecdysone estimated to be present was determined by assay of a sample of the crude extract "spiked" with authentic 20-OH ecdysone (to facilitate identification of this hormone); a total of 263.2 ng was found in a sample of 15 ul, or 17.6 ng/ul (of this, 13 ng/ul was estimated to be the amount from the ticks, representing an estimated 10.87 ng/nymph). Following fractionation, an estimated 69.5 ng of the compound was found in a 5 ul aliquot of the 100% methanol eluate, fraction 3, or 13.9 ng/ul. Therefore, 79.0% of the amount applied was recovered from the cartridge.

Enzymatic hydrolysis did not appear to release any additional 20-OH



ATERS M721 DATA REPORT - EXTERNAL STANDARD QUANTITATION
ANDERS 01/10/84 13:17:32

SAMPLE NAME: VIAL 1 INJECT 1 OF 1
REPORT FILE: NONE PRESSURE: 1123 PSI INPUT LEVEL: 3975

NAME	AMOUNT	RT	AREA	TYP
		2.41	356064	UNK
		2.88	1.26257E+06	UNK
		3.12	1.0551E+06	UNK
		4	67656	UNK
		4.55	225222	UNK
20OHECDYSONE	263.209 NG	5.11	2.7544E+06	STD

Figure 6. HPLC chromatogram illustrating the fractions (compounds?) found in a methanolic extract of D. variabilis engorged nymphs. The polar fractions at RT (retention times) 2.88 and 3.12 are radioimmunoassay positive. Fraction 5 represents natural 20-OH ecdysone co-eluting with the authentic standard.

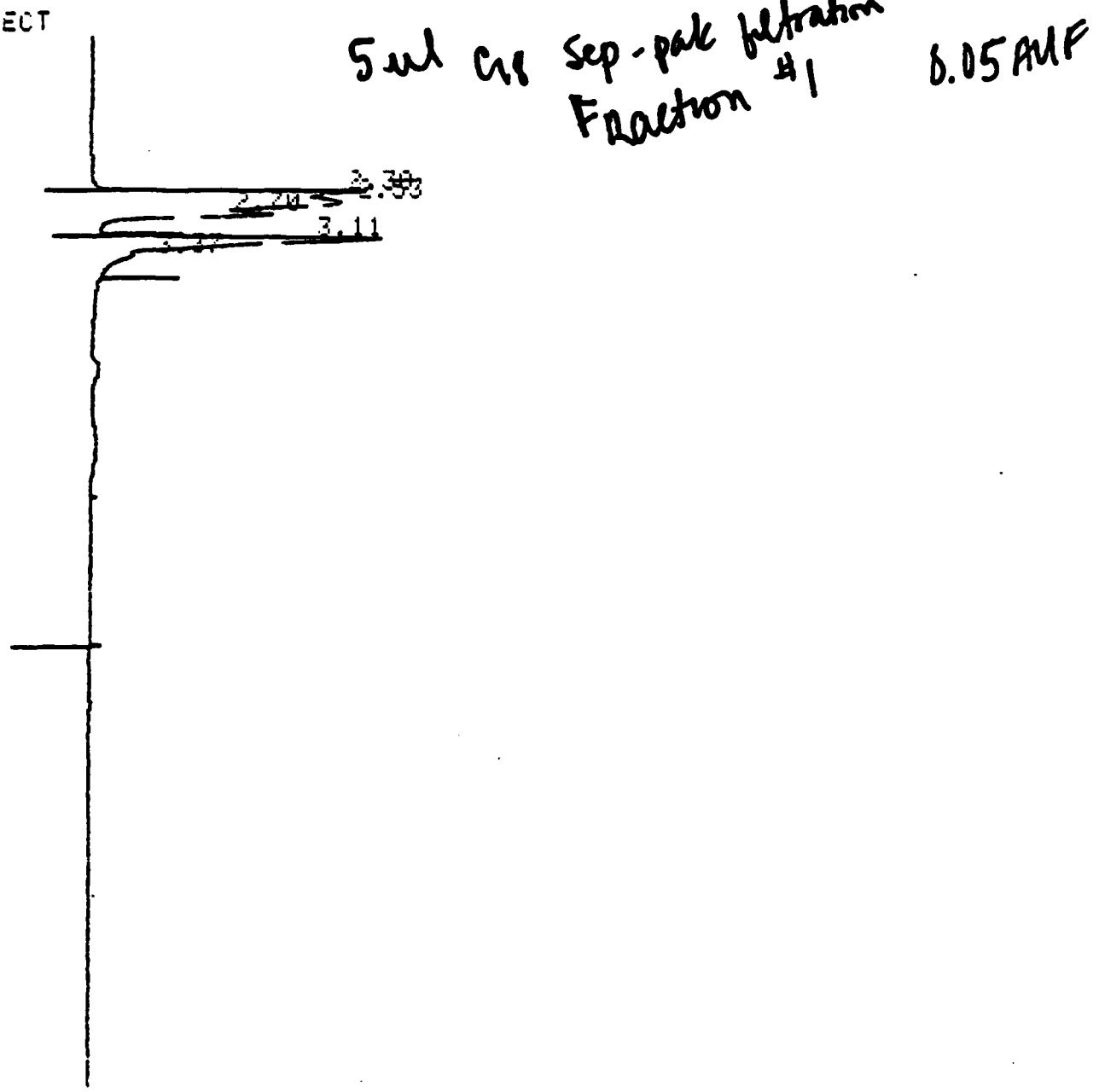
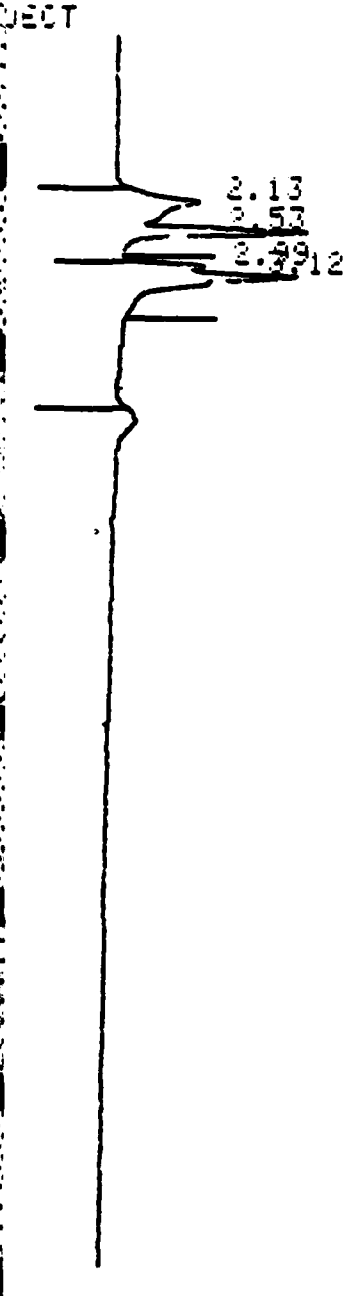


Figure 7-9. HPLC chromatograms illustrating the compounds present in sequential elutions of a C-18 cartridge (Sep Pak) containing the tick extract. 7) 10% methanol elution, polar fractions 1 and 2 present, no 20-OH ecdysone. 8) 30% methanol eution, polar fractions 1 and 2 still present no 20-OH ecdysone. 9) Small quantities of polar fractions 1 and 2 still present, large quantity of 20-OH ecdysone eluted.



5 mL $F_2^{\#}$ C18 pep-pak filtration
0.05 AMP

Figure 7-9. Continued

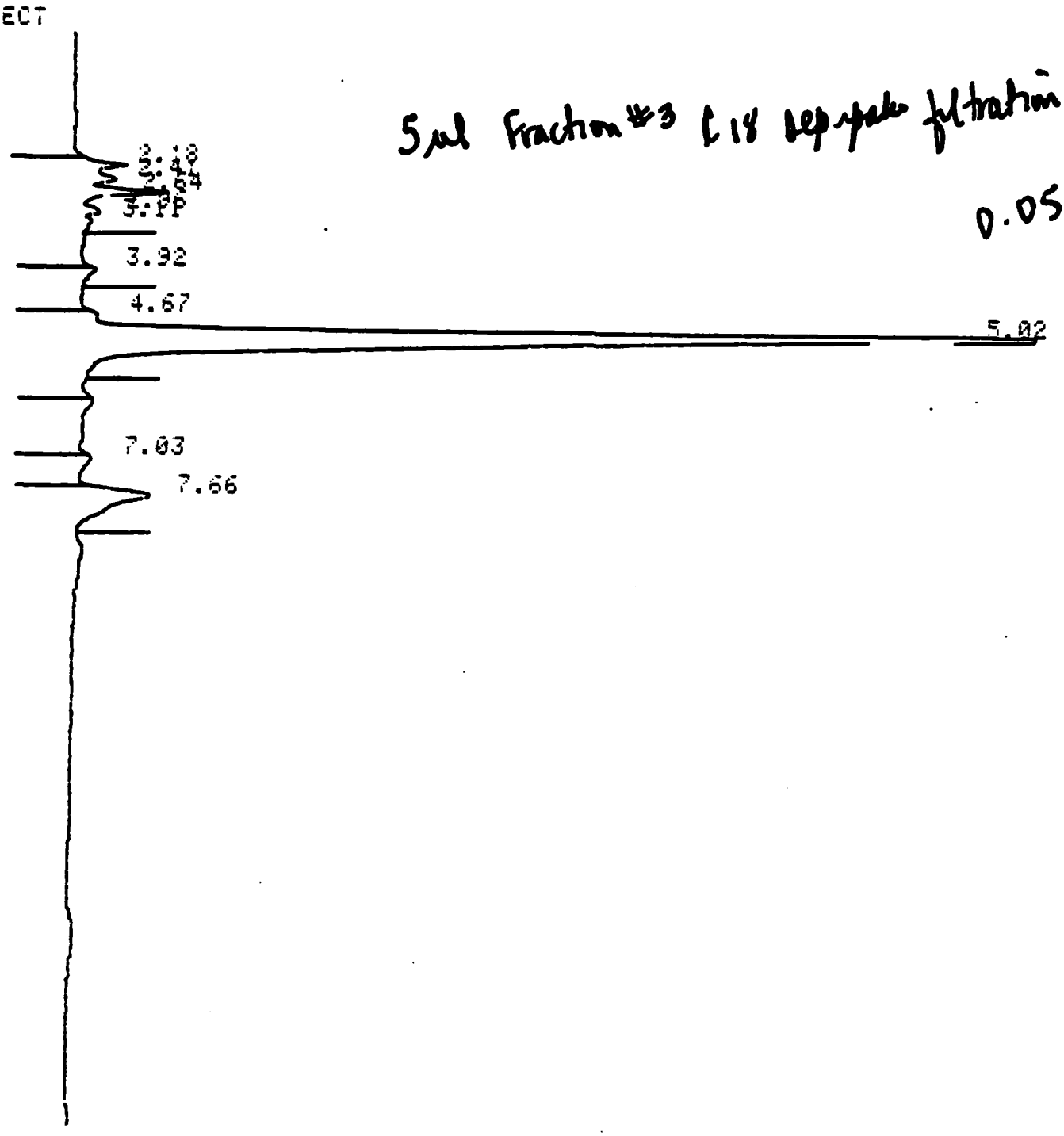


Figure 7-9. Concluded

ecdysone. Assay of a 5 ul aliquot of a sample of the crude extract sujected to enzymatic hydrolysis gave an estimate of 5.04 ng/ul, or 4.21 ng/tick, which is less than the amount estimated to have been present in the crude etract prior to treatment. Clearly, no additional 20-OH ecdysone was released.

Subjecting specific fractions collected from the fractionation of the crude extract by HPLC to enzymatic hydrolysis also did not appear to release significant additional amounts of ecdysone or 20-OH ecdysone. Assay of 7 fractions following the enzyme treatment revealed traces of 20-OH ecdysone in Fraction 3 (retention time 4.24 min. at 55:45, methanol:water, 1 ml/min), but not in any of the other fractions.

The similarity of the polar steroids to the authentic ecdysteroids, ecdysone and 20-OH ecdysone, was confirmed by proton NMR. Excluding the signals for water or solvent, ecdysone has major peaks at +0.85, +0.90, +1.33, and a minor signal at +3.5; 20-OH ecdysone has strong signals at +0.90, +1.33, +3.55, and +4.05 ppm. In contrast, unknown fraction No. 1 has peaks at +0.96, +0.98, +1.00, +1.05, +1.35, +1.42, and +3.65. Unknown No. 2 has peaks at +0.87, +01.22, +1.30, +1.60, +3.48, and +4.25. Finally, unknown No. 3 has a major peak at +1.00, others at +1.32, +1.53 (major peak), a doublet at +1.58, and other peaks at +1.65, +2.13, +2.37, +2.50, +2.65, +3.65, and +4.15. The signals at (1) +0.90 to +1.00, (2) +1.30 to +1.35, and (30 +3.48 - +3.65 are present in all of the compounds and probably represent the same functional groups. In this respect, all of the compounds resemble one another and the authentic ecdysteroids. The 3 unknown compounds have additional signal peaks at (1) +1.53-1.65 and (2) +4.15 to 4.40. The latter is also shared with 20-OH ecdysone (but not with ecdysone) and may represent the C_{22} fraction.

Infrared analysis revealed spectra with absorbance bands resembling those characteristics of steroids (Fig. 11 - 15). Fraction 1 exhibited intense absorption at 1047, 1407 1597-1632, 2929-2957, and 3380 mu, which resembles the spectra of ecdysone and 20-OH ecdysone, as well as the published spectrum for ecdysone (Hoppe et al. 1965). Weak absorbance bands which occur at 2330-2365 mu are absent in the authentic standards. Fraction 2 shows only a weak absorbance band at 1048 and2929 mu, and a strong absorbance band occurs at 1576 rather than 1639. The spectra for fractions 3 and 4 resemble that of fraction 2, with a very weak absorbance band at or about 2942-2964. Fraction 5 (not shown in the figure) exhibited the least resemblance, with strong absorbance bands only at 1428-1442 and 1576. Thus, of the 5 fractions examined and compared with ecdysone or 20-OH ecdysone, fraction 1 appeared most similar. It is almost certainly a steroid. The characterization of the others is less certain, although fractions 2 and 4 are probably steroids also.

Comparison of the mass spectra of the same 3 polar compounds analyzed by NMR revealed a very high degree of similarity between these compounds and the authentic standards, ecdysone and 20-OH ecdysone. No ions larger than 395 amu's were detected. The most abundant ions were in the range from 55 to 129, indicating extensive fragmentation of the molecule. Except for differences in abundance of the various ions (fragments) detected, the spectra of the various compounds were indistinguishable from one another or from the authentic ecdysteroids. Cholesterol gave a very distinctive spectrum, with the most abundant mass at 386, close to un-ionized mass of the molecule, and was readily distinguishable from the spectra of the other compounds. These results suggest that the unknown compounds are probably

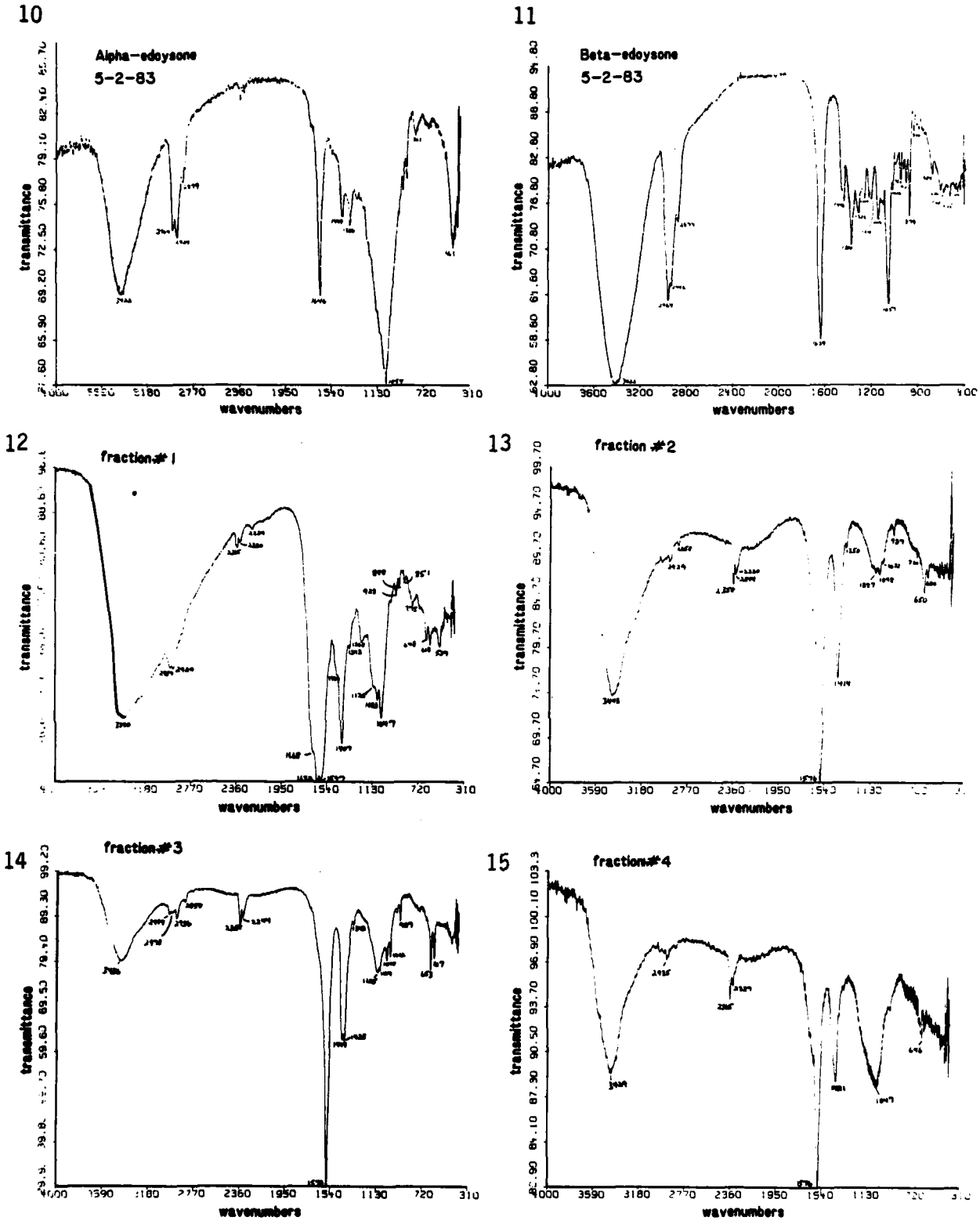


Figure 10-15. Infrared scans of the authentic ecdysteroids, ecdysone (alpha ecdysone) and 20-OH ecdysone (beta ecdysone) and 4 unknown fractions eluted from tick extracts by HPLC.

steroids, similar to ecdysone and 20-OH ecdysone, but precise identification could not be determined from these findings.

Discussion

The results of these studies suggest the presence of steroids other than ecdysone or 20-OH ecdysone in the tick extracts. Analysis of spectra obtained by the methods of NMR, Infrared, and mass fragmentation spectroscopy indicate that the most highly polar compounds are steroids similar to ecdysone, i.e., poly-hydroxylated heterocyclic compounds and readily distinguishable from sterols (e.g., cholesterol). Conjugation of ecdysone or 20-OH ecdysone in these extracts with glucuronic acid or sulfates is excluded by tests with glucuronidase and sulfatase; no significant increase in the hormones was detected when the enzyme treated mixtures were assayed (both RIA and HPLC).

The metabolism of ecdysone has been studied in insects and in ticks. In insects, ecdysone is hydroxylated in the fat body to 20-hydroxyecdysone, the active hormone, and several inactive products, especially 26-hydroxyecdysone, 3-dehydryoxyecdysone, and others (Koolman et al. 1973). Koolman et al. (1973) liberated ecdysone and 20-hydroxyecdysone by treatment of frations in Locusta migratoria extracts with the enzyme glucuronidase. A second fraction, which did not respond to this enzyme, liberated ecdysone, 20-OH ecdysone, 3-dehydroecdysone and 3-dehydroecdysterone when treated with the enzyme steroid sulfatase, indicating the existence of sulfate ester conjugates. Treatment of all fractions with glucosidase or carboxylesterase was ineffective. The authors concluded that the ecdysones were inactivated by conjugation with glucuronic acid and/or sulfuric acid.

In another study, Isaac et al. (1982) found evidence of ecdysteroid inactivation in the form of phosphate conjugates, rather than glucuronates or sulfates, in the desert locust, Schistocerca gregaria. Two highly polar conjugates were found, (1) ecdysone 22-phosphate, and (2) 2-deoxyecdysone-22 phosphate. The structure of these molecules was elucidated by acid hydrolysis of the highly polar compounds, liberating inorganic phosphate, incorporation of ^{32}P into the compound when adiministered by inoculation of ^{32}P ATP or ^{32}P H_3PO_4, into adult locusts, NMR spectroscopy, and Fast Atom Bombardment spectroscopy.

In ticks no evidence of conjugate formation has been reported. Bouvier et al. (1982) tentatively identified one highly polar function in their Ornithodoros moubata extract as 20, 26-dihydroxyecdysone. They suggested several possible pathways for inactivation of ecdysone and 20-hydroxyecdysone; up to 90% of the ^{3}H ecdysone injected into ticks is metabolized to polar and apolar metabolites within 24 hours.

In summary, in ticks, as in insects, the only active ecdysteroid hormones are ecdysone and 20 OH-ecdysone. Various pathways for the metabolism of these hormones have been suggested, all leading to inactivation products that may accumulate in substantial quantities. Further inactivation of metabolites may be accomplished by conjugation in the form of glucuronates, sulfates or phosphates; conjugates may also be used to temporarily inactivate the hormones themselves, i.e., serve for the storage pending later use. No evidence of such conjugates has been found in ticks, and the compounds described here (as in O. moubata) are probably dehydroxy or deoxy forms of ecdysone, e.g., 20, 26-di Oh-ecdysone, 2-deoxyecdysone, or others.

Further study will be necessary to confirm the identity of these polar steroids. Studies using Fast Atom Bombardment, as well as experiments to determine incorporation of radionuclides, are planned.

Table 5. Comparison of NMR spectral signals obtained with authentic ecdysone standards and (presumed) steroid compounds from ticks (D. variabilis).

	Peak No. (δ)													
Identity	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Ecdysone	0.85	0.90	—	—	—	—	—	1.33	—	—	—	—	3.55	—
20-OH ecdysone	—	0.90	—	—	—	—	—	1.33	—	—	—	—	3.55	4.10
Unknown No. 1	—	0.87	0.96	0.98	1.00	1.05	—	1.35	1.42	1.65	—	—	3.65	4.40
Unknown No. 2	—	0.87	—	—	—	—	1.22	1.30	—	1.60	—	—	3.48	4.30
Unknown No. 3*	—	—	—	—	1.00	—	—	1.32	—	1.53	2.13	2.37	3.65	4.15

*Also has minor peaks at +1.58, +1.65, and +2.50.

V. CONTINUED STUDIES ON THE EFFECTS OF ECDYSTEROID ANALOGUES ON DEVELOPMENT AND SEX PHEROMONE ACTIVITY IN *HYALOMMA DROMEDARII*

Introduction

The previous progress report reviewed the effects of an ecdysteroid analogue, BSEA-28 (Thompson et al. 1971) administered to immature *H. dromedarii* feeding on rabbits. Controlled release devices were implanted into the host animals in the expectation of maintaining uniform blood levels of the test compound. Observed effects included reduced body weight, marked reduction in the time required for ecdysis, and excitation of sex pheromone activity.

Solomon et al. (1982), in their review of the potential for hormonal control of tick infestations on domestic animals, concluded that there was little reason for optimism regarding the use of ecdysteroids. This perspective has now changed as a result of the work on Connat et al. (1983) with the ecdysteroid, 22, 25 didexoyecdysone (DDE, or BSEA-1). These workers oserved accelerated molting in *Ornithodoros moubata* with as little as 35 ng/ml of blood containing DDE, and substantial mortality in this species when the concentration of DDE was increased to 500 ng/ml of blood. These findings offer much promise for the use of this highly potent analogue in the control of ticks.

This brief report describes continuing studies with the ecdysteroid analogue, BSEA-28, to assess its effects when applied in different amounts, and its persistence in the host. The same slow release delivery system, i.e., implantation of the active compound in plastic tubes sutured under the host skin, described in the previous progress report was used throughout this study.

Materials and Methods

BSEA-28 (=Beta, 5 beta-14-aplha-trihydroxy-5 beta-cholest-7-en-6-one) was synthesized by published methods (Thompson et al. 1971), and was donated as a gift from Dr. Thompson (via Dr. Jaffe, USDA, BARC, Beltsville, MD.). The material was suspended in ethyl oleate (25%, w/w of active ingredient, AI) and deposited in tubes of the permeable plastic, polycaprolactone. The filled tubes were cut into 4-cm lengths and sealed with silastic plugs. The resultant 4-cm tubes each contained an estimated 26 mg AI.

Implantation of the devices containing the ecdysteroid analogue was done by suturing them into the shoulder and back areas of normal, adult rabbits, Oryctalagus cuniculus, as described in the previous progress report. The first replication of the treatment was done with 3 devices implanted into each of two rabbits, 3 devices/animal, for a total of ca. 77 mg AI/rabbit. One day after implantation of the devices, ca. 300-400 laboratory reared H. dromedarii larvae, ca. 3-6 weeks old, were released onto the animals and confined with the aid of feeding capsules. To facilitate recovery of the engorging nymphs, the feeding capsules were replaced during the infestation period, when the larvae had molted and the nymphs reattached (this is a 2-host species) with stockinet. The infested animals were monitored regularly for evidence of feeding and molting of the immatures, and recovery of the engorging nymphs. The engorged nymphs that dropped were placed in vials dated for the day of drop off and held in the Aminco Climate Chamber at 80 ± 1° F and 92 ± 2% RH. Six weeks later, after all attached ticks had dropped or died, the same animals were infested again with ca. 900-1000 larvae/animal, and the observations of tick feeding, and development repeated.

Blood samples were collected from the rabbits with the BSEA-28 implants to be used for determination of detectable BSEA-28 in the circulating blood of these animals. Samples were collected at weekly intervals from the animals, extracted in accordance with methods described by Dees et al. (1984), and held for analysis by RIA and HPLC.

To determine whether fecundity or fertility of the ticks exposed to the BSEA-28 treatments was affected, adults from the treated populations were selected at random and allowed to feed and mate on a normal host rabbit. Following mating and repletion, 5 replete females from each of the 2 populations were selected for these determinations. Total engorged weight and egg mass weight were measured in each case; hatching success was estimated in each case.

Studies of the efffects of the ecdysteroid analogue (BSEA-28) on sex pheromone activity in the emerged adults were contiuned, using the same procedures described by Dees et al. (1984) and in previous progress reports. The only change was the substitution of a capillary column (DB-1), instead of the packed columns used in previous studies.

Results

Table 6 summarizes the results of the various tests with devices containing this compound implanted into rabbits. The table includes data reported in the previous progress report, incorporated here so as to furnish a more complete review of the findings. The first test was BSEA-28 delivered in this mode (controlled release implants) with approximately 48 mg/animal indicated highly significant reductions in engorged nymphal weight, and accelerated molting (treatment no. 1). The yields of viable engorged nymphs

Table 6. Effect of presence of ecdysteroid or IGR implanted into host rabbits on engorgement weight and molting in Hyalomma dromedarii.

Type of Comp. Implant	Treat-ment No.	Wt. Mater. (mg.)	Wt. of Eng. Nymphs No.	Wt. (mg.)	No. Fed	No. Live	MOLTING Molting Dead	Duration (Days)
Methoprene	1	41.9	48	20.92 ± 4.08	72	71	1	22.19 ± 1.76
"	2	81.3	45	32.98 ± 4.65	102	101	1	22.97 ± 1.89
Ecdysterone	1	190.0	87	26.40 ± 4.87	199	193	6	20.39 ± 2.41
BSEA-28	1a	47.6	6	21.93 ± 4.69	6	6	0	———*
"	1b	47.6	25	13.53 ± 4.79	75	73	2	15.43 ± 3.78**
"	2a	77.8	51	26.40 ± 6.20	152	150	2	19.75 ± 2.35
"	2b	77.8	51	20.96 ± 4.60	160	154	6	17.66 ± 3.32**
6 weeks	2a	77.8	60	14.20 ± 0.34	635	549	93	16.62 ± 3.98**
6 weeks	2b	77.8	60	13.00 ± 0.59	583	566	17	19.35 ± 1.90
BSEA-28	3a	389.0	80	8.00 ± 0.34	136	136	0	18.01 ± 1.43**
"	3b	519.0	100	11.50 ± 0.30	591	588	3	17.86 ± 2.20**
Propylene glycol		37.5	30	21.66 ± 4.67	74	72	2	21.35 ± 2.06
Untreated control	—		24	20.53 ± 3.94	66	66	0	20.34 ± 1.68
Untreated Control	—		100	18.24 ± 5.40	634	632	2	19.84 ± 2.11

*Only 6 specimens.

**Significantly different from the controls by the student's T test, $P < 0.01$.

were also reduced, especially in the case of treatment no. 1a. When the test was repeated with ca. 77 mg/animal, significant acceleration of the ecdysial period was observed in the ticks that fed on one of the treated animals but not the other, and no reduction in body weight was observed in the ticks from either animal. When these same animals were infested again 3 weeks later, or 6 weeks after installation of the devices, the results indicated highly significant reductions in body weight in the tick population from both animals, and a significant acceleration of molting in the ticks from one of the animals [$2a_2$] but not the other. The yields of viable engorged nymphs were high in both cases, ca. 60% of the estimated 900-1000 larvae having transformed into engorged nymphs. However, unusually high mortality was observed in the ticks from treatment $2a_2$.

The results of the third treatment, done with 519 and 389 mg/rabbit, respectively, implanted into the host animals, revealed highly significant reductions in nymphal engorgement and accelerated development. The results of treatment 3b, with 519 mg/rabbit, are especially noteworthy. Not only was the ecdysial period shortened significantly when compared to the controls, but many individuals molted rapidly, one in as little as 6 days. None of the individuals in any of the 3 control groups molted in less than 15 days. Numerous very small nymphs were observed in the ticks exposed to the treated animals; 32 of 100 individuals weighed were less than 10 mg on the day of drop off. Treatment 3a, with 389 mg/rabbit, resulted in the most extreme reduction in engorged tick body weight ever recorded, and the total yield, ca. 14% was much less than with all but one of the preceding treatments (1b). Figures 16 and 17 illustrated the frequency distributions of individual tick molting times in days.

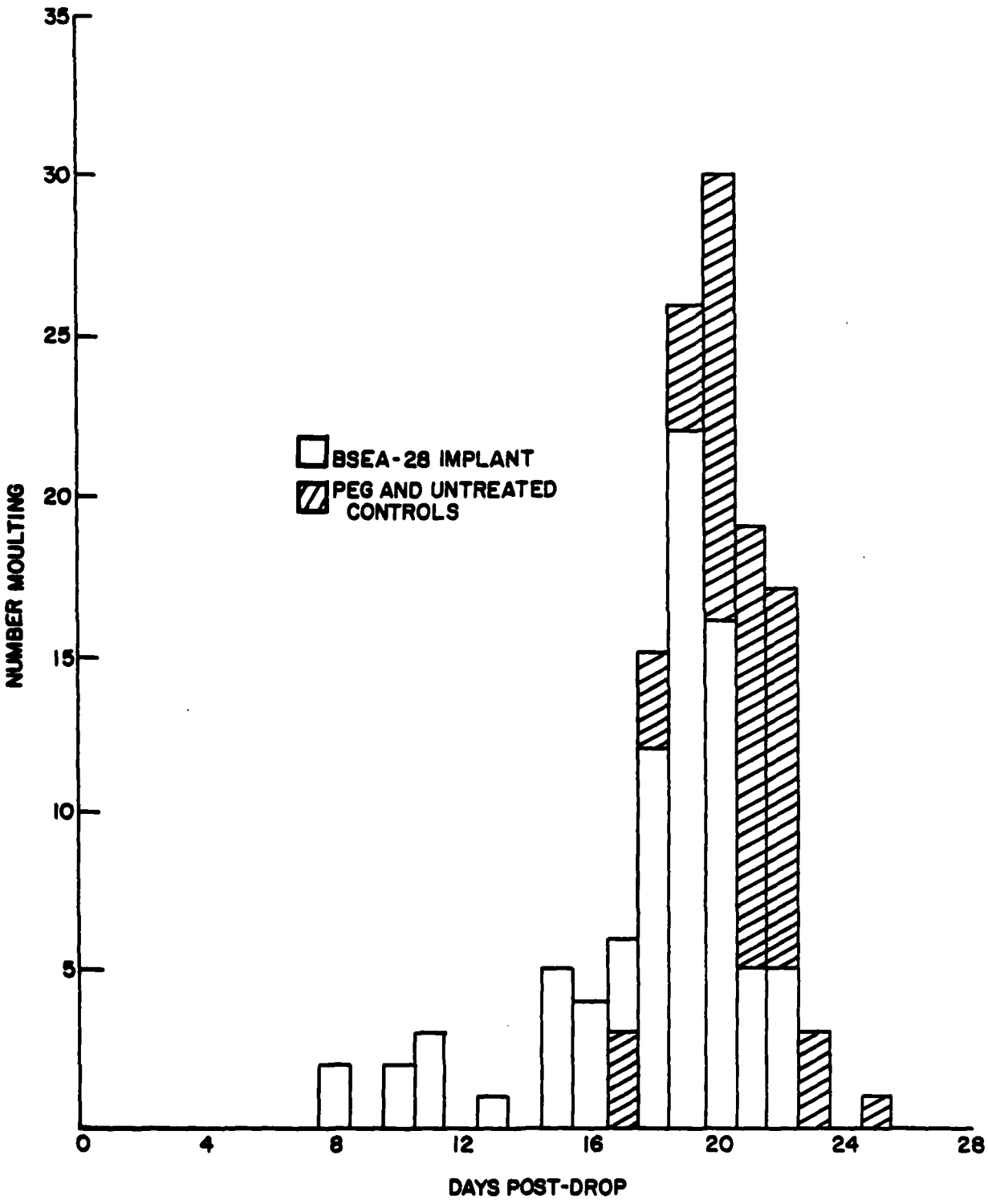


Figure 16. Frequency distribution comparing the numbers of H. dromedarii engorged nymphs molting on different days following feeding on rabbits with BSEA-28 versus controls. Data for rabbits with 3 implants.

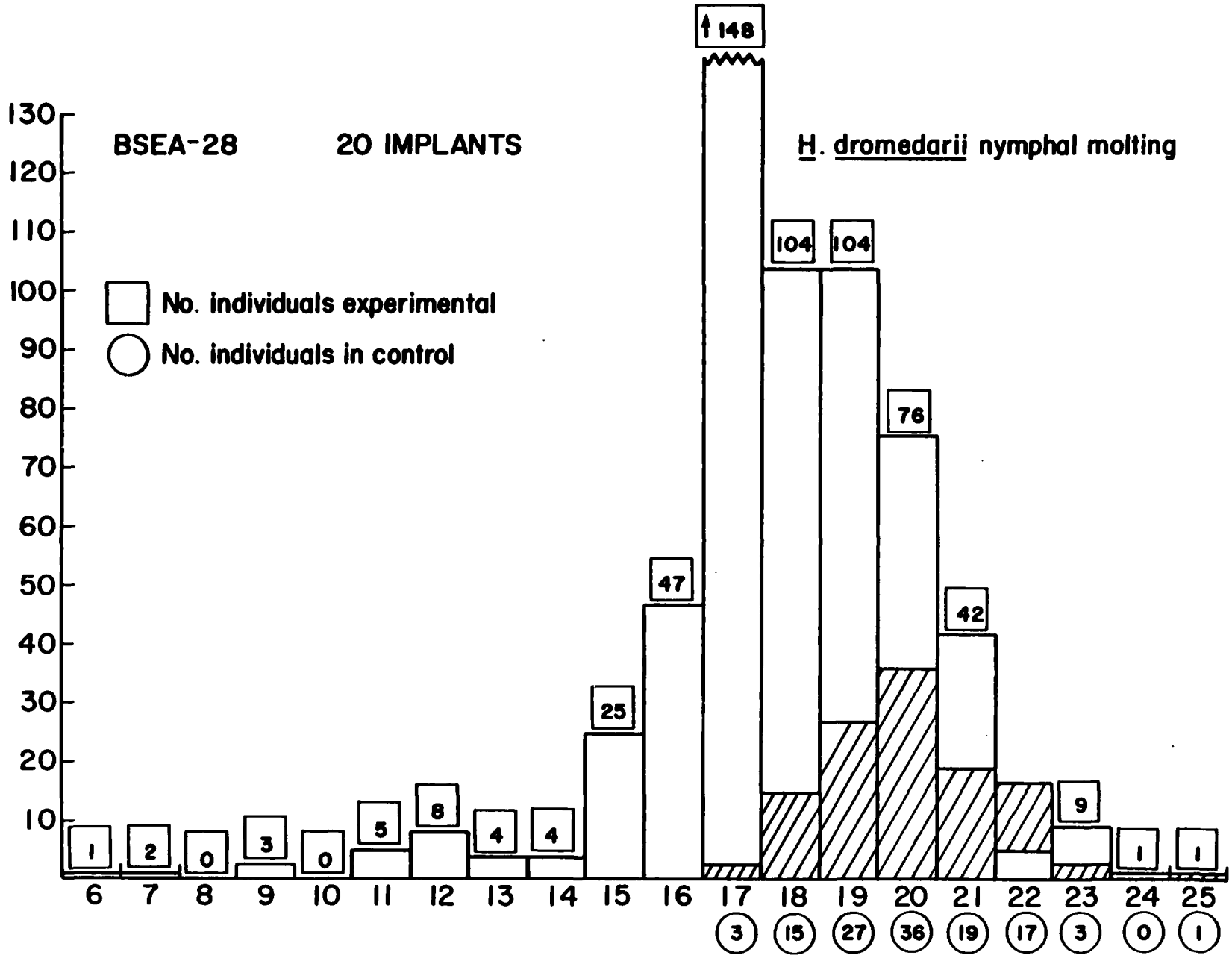


Figure 17. IBID. Data for rabbit with 20 implants.

Discussion

Efforts by Mango (1979) and her co-workers to disrupt tick development by treatment with ecdysone, 20-hydroxyecdysone, or ponasterone, were successful, but only when abnormally high concentrations of these hormones were applied to the ticks. These findings, reviewed by Solomon et al. (1982), led to a rather pessimistic evaluation of the potential of these steroid hormones for use in the control of tick infestations on livestock. However, Solomon et al. did not totally preclude this potential use, and noted that this strategy may merit reevaluation if a more potent steroid analogue could be developed.

The findings reported in this study suggest that the analogues of the BSEA series may provide the desired potent analogues of 20-hydroxyecdysone noted above. Studies with the analogue 22, 25 dideoxyecdysone (DDE), also known as BSEA-1, by Connat et al. (1983) are certainly consistent with this hypothesis. The results with BSEA-28 reported in this study are also consistent with the effects observed elsewhere, namely, accelerated development, reduced body weight, and, in some cases, considerable mortality. However, the effects observed were marginal, except when exceptionally high concentrations of the active ingredient were used, and several inconsistencies are apparent in the data. Although the results of the first treatment with ca. 48 mg/animal led to impressive reductions in the amounts of blood imbibed and time required for molting, implantation of ca. 78 mg/animal led to accelerated molting in the ticks fed on only one of the two animals, and no significant reduction in blood imbibition. Reinfestation of the same animals 6 weeks later resulted in the expected blood imbibition reductions, but molting time was reduced in only one of the populations. This may

indicate delayed release of the AI, rapid encapsulation of the implanted devices with fibrous tissue, retarding diffusion of the AI into the general circulation, or other, unknown effects. Clearly, interpretation of the results is hindered by the lack or knowledge of the precise blood levels to which the ticks were exposed, in contrast to in vitro studies where precise amounts were administered.

Table 7. Occurrence of 2,6-dichlorophenol in Hyalomma dromedarii adults that emerged from nymphs exposed to different hormonal treatments.

Treatment	Sex of Ticks	No. of Individuals	2.6-DCP ng/DCP
Ecd. inocul. 10 ng/nymph	females	68	13.09
"	males	26	1.97
Ecd. inocul. (10 ng/nymph) + 10 ug JH	females	71	9.69
"	males	43	3.52
BSEA-28 implant	females	49	10.10
"	males	45	5.03
Ecdysterone implant	females	100	5.80
"	males	105	0.00
Methoprene implant	females	89	6.90
"	males	114	0.00
Untreated control	females	to be done	5.57*
"	males	to be done	0.01*

*From Sonenshine et al. (in press, J. Med. Entomol. 20(4):000-000), with the permission of the editor.

Table 8. Fertility and fecundity of ticks, H. dromedarii, emerged/from nymphs fed on BSEA-28 (implants) treated rabbits

Treatment	Wt. Engorged $\bar{X}$ ± S.D. (mg)	Wt. Eggs $\bar{X}$ ± S.D. (mg)	Total No. Eggs $\bar{X}$ ± S.D.	Estimated Hatching
BSEA-28 519 mg	880 ± 172	360 ± 86	7,718 ± 1855	> 98%
BSEA-28 389 mg	694 ± 183	276 ± 168	5,931 ± 1798	> 98%

VI. STUDIES ON ECDYSTEROIDS IN THE ARGASID TICK, ORNITHODOROS PARKERI.

Introduction

Semi-annual progress report on work completed at Georgia Southern College on ONR Project "Hormonal-Pheromonal Interrelationships in Ticks and Parasitic Mites," (Contract N00014-80-C-0546). James H. Oliver, Jr., Co-Principal Investigator (subcontractor).

Parabiosis

In the last progress report we confirmed a regulatory relationship between beta-ecdysone and apolysis in Ornithodoros parkeri, and noted the potential use of this system as a new readily available and inexpensive bioassay for ecdysterone. Experiments involving molting in ticks have continued and attempts were made to show "physiological" presence of ecdysones in tick hemolymph via experiments involving parabiosis. Parabiosis of ticks was performed by joining "donor" tick to "recipient" tick with a minute glass capillary tube inserted into the stumps of the coxae IV. In experiment 1 the "donors" and "recipients" were joined prior to allowing the "donors" to feed (recipient ticks not allowed to feed). In experiment 2 the "donors" were fed 24 hours prior to joining them to unfed "recipient" ticks. None of the unfed nymphs which were joined to the fed nymphs in both experiments molted. Nevertheless, the fed ticks parabiosed to unfed ones molted; although they required more time than nonparabiosed controls. Failure of "recipient" parabiosed ticks to molt prompted experiment 3 in which two "donors" were joined to each "recipient" in an attempt to supply greater amounts of the presumed ecdysone in the hemolymph to unfed "recipients." Unfortunately, the unfed recipients did not ecdyse, but the donor ticks did ecdyse as in the aforementioned experiments. Interestingly, double molting

occurred at a significant level in fed donors ($p<0.001$) and in fed sham operated controls ($p<0.01$). Apparently surgical trauma prompted additional ecdysone production. Experiment 4 consisted of joining each fed virgin female (as recipients) to 2 fed fourth stage nymphs (5 days post-fed). None of the females ecdysed, although the "donor" nymphs molted after a delay.

Ligation

Attempts were made by use of ligatures to determine the general location of the tissues which produce molting hormone. A primary problem with most attempts to ligate ticks, however, is that the essentially nonsegmental body plan of Ornithodoros parkeri does not lend itself to ligation, and the principle effect of ligation is death. Even when ligations were applied less tightly the principle effect was mortality, although some cases of complete molting occurred (i.e. both anteriorly and posteriorly to the ligature).

Information was obtained from histological sections of some ligated ticks. Examinations of these sections revealed that cuticular changes in the posterior body portions were usually further advanced in the molting process than they were in the anterior portions. In most cases only the posterior portion showed any signs of molt related changes. This pattern of molting activity occurred whether the synganglion had been isolated anteriorly or posteriorly to the ligature, indicating that the molting hormone was not produced by the synganglion as suggested by Cox (1960), but by a tissue posterior to the synganglion. These results were unexpected because Cox observed partial molting of Ornithodoros turicata and thought that molting was restricted anteriorly to the ligature. Some of the ligated D. parkeri

in our experiments also appeared to molt anteriorly to the ligature, but when the ligature was removed it was seen that the posterior portion of the exuvium was loose, viz. what appeared to be partial anterior molting was in fact mechanical retention of the exuvium by the ligature. These experiments should be repeated to resolve the apparent differences between the two studies. Although the peritracheal glands were not positively identified in the present study, they are described as being "associated with the tracheal plexus ventral to the central nerve mass..." (Roshdy and Marzouk, 1982), i.e. this should place them anteriorly to the ligature in experiment 3. Our results suggesting that some tissue posteriorly to the synganglion produces the molting hormone also do not support the hypothesis that the peritracheal glands are the producers of the molting hormone.

Synganglial Transplants

If the synganglion or a closely associated tissue produces a molting hormone, transplants of nymphal synganglia taken at our just before a presumed peak in hormone production might cause a molt in an adult. Therefore, synganglia of Ornithodoros parkeri third stage nymphs (FN3)s were transplanted into adult females in an attempt to induce supernumerary molts.

Fed N3s served as synganglial donors and virgin females as recipients. Fifty-eight FN3s 4 days post-feed (10 groups of 5 and another group of 8) and 30 virgin females 4 days post-feed were divided into groups by weight immediately prior to surgery. One group of 10 females received 4-5 FN3 synganglia each and 2 groups of 5 females served as non-synganglial tissue controls. Five received muscle (both gnathosomal and opisthosomal) implants and 5 received 1 1/2 - 2 salivary glands. Untreated controls were 8 FN3s and 10 virgin females.

No molting was observed among any females receiving synganglial transplants from N3s, and upon dissection all females had only one cuticular layer. All control N3s molted normally and, as expected, females receiving muscle or salivary glands and untreated control females failed to molt. Transplanted synganglia showed no evidence of deterioration or encapsulation upon dissection of recipient females 30 days after implantation.

Synganglial transplants performed 4 days post-feed would be expected to provide tissues at or just before peak molting hormone production if ecdysteroids are produced at the same time in O. parkeri as they are in O. p. porcinus and O. moubata. Ecdysteroid titers peak on day 4 in O. p. porcinus (Mango and Moreka, 1979) and day 5-6 in O. moubata (Germond et al., 1982). Since none of the ticks receiving the transplants molted and the transplanted synganglia appeared normal without signs of deterioration or encapsulation after 30 days, it may be inferred that they do not produce ecdysone or were not producing it at the time they were extirpated. Future experiments using timed series of synganglial transplants beginning shortly after feeding and continuing through day 4 post-feed and later would allow a better comparison with synganglial induced molting observed in O. turicata (Cox, 1960). In experiments with O. turicata, 2 adult females and one adult male were induced to undergo supernumerary molts after receiving 3-4 synganglia from FN3s 48-72 hours post-feed. All subsequent attempts by Cox to repeat this experiment were unsuccessful.

Injection of Crude Synganglial Extract

If the synganglion or its closely associated tissues produce molting hormone, then crude extracts might induce supernumerary molts in adults. A crude synganglial extract was prepared by excising the synganglia of 30

Ornithodoros parkeri FN3s (24-48 hours post-feed) and placing them in a small conical vial made from the tip of a glass Pasteur pipet. The vial was kept cold (ca. 0°C) in an ice bath during this time. Gnathosomal and opisthosomal muscle bundles excised from the same ticks were placed in a second vial. When all the tissues had been excised they were macerated with a glass rod and subjected to ultrasonic vibration by immersing the tip of the vial in a sonicator (Mettler Electronics Corp., Anaheim, CA) for two 1-minute periods.

Centrifugation of the macerated tissues of 0°C (900x g) for 15 minutes yielded 2.5 µl of synganglial supernatant and 2.0 µl of muscle supernatant. Vials were kept on ice until used (synganglion 5 minutes, muscle 70 minutes).

Recipient ticks were virgin males which had been divided into 4 weight equivalent groups of 10 ticks each. Three synganglial recipients received 0.4 µl of the extract (5 tick equivalents) and 6 received 0.2 µl (2.5 tick equivalents). Three muscle homogenate recipients received 0.3 µl (4.6 tick equivalents) and 7 received 0.15 µl (2.3 tick equivalents). Saline injected males received either 0.4 µl (4 ticks) or 0.2 µl (6 ticks) of Shen's saline. Controls were 10 untreated males.

Neither molting nor mortality were observed among males receiving injection of synganglial extracts from nymphs. Moreover, none of the control males molted.

Male O. parkeri were chosen as bioassays for injections of crude synganglial extracts from FN3s because O. p. porcinus males were found to be more susceptible to lethal effects of exogenous ecdysteroids than females; moreover, higher numbers of males began to molt in response to treatment with B-ecdysone although more females molted completely (Mango, 1979).

Since neither mortality nor molting of O. parkeri males occured in response to injections of crude synganglial extract is seems likely that the synganglia do not produce molting hormone or were not at the proper physiological state. Improper timing could be eliminated by a timed series of extractions or by first determining molting hormone titer peaks for O. parkeri chemically and then injecting.

VII. SUMMARY

The studies described in this report have contributed new evidence of hormone-pheromone interactions, as well as new information on the hormone profile of ticks. In general, ticks resemble most other arthropods, with only one active ecdysteroid, 20-OH ecdysone, and (thus far) only one juvenile homrone, JH 3.

The major emphasis during the current project period was directed to the natural tick hormones and their interaction with sex pheromones. JH 3, the most common of the juvenoids found in insects, was identified in feeding virgin females of H. dromedarii and D. variabilis by a highly specific radioimmunoassay done at the Universite Pierre et Marie Curie, Paris, France; estimates of the hormone quantities were 76.7° 3.1 pg/female for H. dromedarii and 3.4° 1.3 pg/female for D. variabilis. No evidence of JH 1 or JH 2 was found by this method. The results suggest the presence of a juvenoid serving as a gondatropic hormone. Evidence of digestion of JH to polar end products, presumably diols or acids, was also obtained, indicating enzymatic digestion by esterases.

The major site of ecdysteroid synthesis (or storage) was found to be the synganglion and the lateral nerve plexus containing the lateral segmental organs. Surveys of various body organs in feeding virgin female H. dromedarii and D. variabilis demonstrated nanogram quantities in the synganglion and associated nerve plexi, and in the hemolymph. Much smaller quantities were in other organs, probably due, at least in part, to unavoidable contamination with hemolymph. The amounts found in the foveal glands,

however, were unusual when considered on the basis of amounts per milligram of tissue. The foveal glands ranked second, after the central nervous complex, in importance as a site of ecdysteroid accumulation. These findings provide additional evidence of ecdysteroid accumulation in these important pheromone glands. Other evidence of synganglion involvement in ecdysteroid synthesis was obtained with radiotracers. Following inoculation of ^{14}C cholesterol into H. dromedarii, intense accumulation of radiolabelled material were found in the synganglion and lateral nerve plexus by autoradiography; no other organ or tissue exhibited such intense dense concentrations, although significant accumulations were found in the integument and the foveal glands.

New studies described in this report indicate that the highly polar RIA positive compounds noted previously are steroids, and probably represent by-products of ecdysone or 20-OH ecdysone metabolism. They may be further inactivated by forming conjugates. ^{14}C incorporation was observed in these polar compounds following inoculation of ^{14}C cholesterol. However, hydrolysis with glucuronidase, glycosidase, or sulfatase failed to alter these polar compounds or release additional 20-OH ecdysone, presumably ruling conjugates with glucuronic acid, glycosides, or sulfates. Tests for phosphates are yet to be done. Proton NMR and Infrared spectroscopy strongly suggest compounds resembling steroids, but precise identifications could not be determined by these techniques. Attempts to identify the compounds by comparison of their mass spectra with that of an authentic standard were unsuccessful, apparently due to the thermal instability and rapid disintegration of these molecules; no ions resembling the parent ions were obtained. We conclude that these compounds are steroids which, in view of their

high polarity, are probably steroid conjugates or steroid acids resulting from metabolism of ecdysone and/or 20-OH ecdysone by natural enzymes. Further studies to clarify the identity of these highly polar steroids are in progress.

Studies at GSC confirmed a regulatory relationship between 20-hydroxyecdysone and apolysis in the soft tick, Ornithodoros parkeri. New methods were developed to provide convenient and inexpensive but sensitive bioassays for ecdysteroid activity in this model species. One method which shows considerable promise uses parabiosis; other methods used ligation, synganglial transplants, and injection of synganglial extracts. All were done to determine whether ecdysteroids could be transferred from donor to recipient individuals and identify the organ(s) responsible for ecdysteroid production. The findings obtained by these innovative, experimental methods are compared with those of other workers, and their implications for our understanding of hormonal regulation of physiological processes in ticks are discussed.

VIII. FUTURE PLANS

The primary focus of the work at Old Dominion University is being directed to studies on the existence of juvenile hormones in ixodid ticks and their role in sex pheromone regulation. New evidence of JH occurrence in ticks, reported elsewhere (in this report), justifies more extensive studies of the possible interaction between juvenoids and sex pheromones. Studies will be directed to examination of the effects of JH III on sex pheromone synthesis, concentration, and release. Previous tests with JH analogues have given mixed results. In future studies, treatment will be done with JH III administered topically to engorged nymphs, and containing sufficient ^{3}H tracer to determine penetration of the active ingredient through the cuticle. Treatments will also be administered by inoculation in olive oil (or other suitable media). Gas chromatographic assays will be done to measure 2,6-dichlorophenol concentration. If possible, implants will be used to control release of JH analogues in hosts of H. dromedarii as a means of exposing these ticks to these bioactive compounds.

Precursors, labelled with ^{3}H or ^{14}C, will be used to trace JH III synthesis as well as aid in the identification of possible sites of JH production in the tick body. Special attention will be given to the synganglion, where a variety of neuro-endocrine centers have been identified. HPLC will be used to collect the fraction expected to contain JH III for radioassay (thereby determining whether incorporation of the radio label has occurred), while autoradiography will be used to monitor tick body tissues for sites of uptake and accumulation of such activity. These studies are expected to facilitate development of rational control programs, i.e., based on precise knowledge of synthesis, storage and use of this master regulatory agent.

Studies currently in progress on the characterization of RIA positive fractions believed to be metabolic by-products of ecdysteroid metabolism (as free steroids or conjugates) will be continued and completed during the next project year. Such information is important, since it suggests that ticks utilize only a single ecdysteroid, namely, 20-hydroxyecdysone (=beta ecdysone) as the active hormone; other steroids which resemble ecdysteroids are probably inactive metabolites.

Studies at Georgia Southern College on site(s) of ecdysteroid synthesis and storage in Ornithodoros parkeri will be continued. Synganglial extracts and various types of tissue transplants provide convenient tools for monitoring the presence of active hormone when transferred to responsive recipient individuals. These studies provide useful biological models for assessing hormone activity. Similar studies on JH-like compounds, acting as regulators of developmental processes and gonadotropic hormones, will also be continued.

IX. PUBLICATIONS AND MANUSCRIPTS

The following is a listing of all of the papers, published or in press, and manuscripts submitted for publication, produced by project personnel. New listings, produced since the last progress report, are cited first, and identified with an asterisk to the left of the citation. A total of thirty (30) papers have been produced with support from this contract. The following is a comprehensive listing of all work supported by the contract.

I. New citations representing articles or manuscripts generated after May 1, 1983, or never previously cited.

*1. Sonenshine, D.E., Paul J. Homsher, M. Beveridge and W.H. Dees, Occurrence of ecdysteroids in specific body organs of the camel tick, Hyalomma dromedarii and the American dog tick, Dermacentor variabilis, with notes on their synthesis from cholesterol. Submitted to J. Med. Entomol. May 21, 1984.

*2. Sonenshine, D.E. Pheromones and other semiochemicals of the Acari. Annual Review of Entomology (in press) Major review article scheduled for volume 30, 29 pp. (estimated).

*3. Dees, W.H., D.E. Sonenshine and E. Breidling. 1984. Ecdysteroids in the camel tick, Hyalomma dromedarii, and comparison with sex pheromone activity (Acari:Ixodidae). J. Med. Entomol. (in press) 21:000-000.

*4. Dees, W.H., D.E. Sonenshine, and E. Breidling. 1984. Ecdysteroids in the American dog tick, Dermacentor variabilis (Acari:Ixodidae), during different periods of tick development. J. Med. Entomol. (in press) 21:000-000.

*5. Khalil, G.M., D.E. Sonenshine, H.A. Hanafy, and A.E. Abdelmonem. 1984. Juvenile hormone I effects on the camel tick, Hyalomma dromedarii (Acari:Ixodidae). J. Med. Entomol. 21:000-000.

*6. Hagan, D.V., J.H. Oliver, Jr., and J.M. Pound. A morphological study of the coxal organs in adult Ornithodoros parkeri (Acari:Argasidae). Proc. VI Int. Cong. Acarol. 1982, Edinburgh, Scotland, (in press).

*7. Pound, J.M. and J.H. Oliver, Jr. Morphology of the retrocerebral organ complexin penultimate nymphal and adult female Ornithodoros parkeri (Cooley) (Acari:Argasidae). Proc. VI Int. Cong. Acarol. 1982, Edinburgh, Scotland, (in press).

*8. Campbell, J.D. and J.H. Oliver, Jr. Membrane feeding and developmental effects of ingested B-ecdysone on Ornithodoros parkeri (Acari:-Argasidae) Proc. VI Int. Cong. Acarol. 1982, Edinburgh, Scotland, (in press).

*9. Pound, J.M., J.H. Oliver, Jr. and R.H. Andrews. Induction of apolysis

and cuticle formation in female Ornithodoros parkeri (Acari:Argasidae) by hemocoelic injections of B-ecdysone. J. Med. Entomol. (in press).

*10. Pound, J.M., J.H. Oliver, Jr. and R.H. Andrews. Effects of temperature and tick weight on expression of autogeny in the argasid tick Ornithodoros parkeri (Acari:Argasidae). J. Parasitol. (in press).

*11. Severino, G., J.H. Oliver, Jr. and J.M. Pound. Synganglial and neurosecretory morphology of the chicken mite Dermanyssus gallinae (Mesostigmata:Dermanyssidae). J. Morphol. (in press).

*12. Oliver, J.H., Jr., J.M. Pound and R.H. Andrews. Induction of egg maturation and oviposition in the tick Ornithodoros parkeri (Acari: Argasidae). J. Parasitol. (in press).

*13. Oliver, J.H., Jr., J.M. Pound and G. Severino. Evidence of a juvenile hormone-like compound in reproduction of Dermanyssus gallinae (Acari: Dermanyssidae). J. Med. Entomol. (in press).

II. Publications cited in previous progress reports.

1. Sonenshine, D.E., P.J. Homsher, G.M. Khalil, and S.N. Mason. 1982. Dermacentor variabilis and Dermacentor andersoni: genital sex pheromones. Exper. Parasitol. 54:317-330.

2. Khalil, G.M., D.E. Sonenshine, O.A. Sallam, and P.J. Homsher. 1983. Mating regulation and reproduction isolation in the tick Hyalomma dromedarii and Hyalomma anatolicum excavatum (Acari:Ixodoidea: Ixodidae). J. Med. Entomol. 20(2):136-145.

3. Silverstein, R.M., J.R. West, D.E. Sonenshine, and G.M. Khalil. Occurrence of 2,6-dichlorophenol in the hard ticks Hyalomma dromedarii and Hyalomma anatolicum excavatum and its role in mating. J. Chem. Ecol. (in press).

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5. Khalil, G.M., S.H.A. Gaber, D.E. Sonenshine, and S.M. Gad. IBID 2. Larval and nymphal responses. J. Med. Entomol. (in press).

6. Khalil, G.M., A.A.A. Shaarawy, D.E. Sonenshine, and S.M. Gad. B-Ecdysone effects on the camel tick Hyalomma dromedarii. J. Med. Entomol. 21:188-193.

7. Khalil, G.M., D.E. Sonenshine, P.J. Homsher, W.H. Dees, K.A. Carson, and V. Wang. 1983. Development, ultrastructure and activity of the foveal glands and foveae dorsales of the camel tick, Hyalomma dromedarii (Acari:Ixodidae). 1. Primordial growth and gland function. J. Med. Entomol. 20:414-423.

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9. Dees, W.H., D.E. Sonenshine, E. Breidling, and G.M. Khalil. 1982. Toxicity of precocene-2 for developmental stages of the American dog tick, Dermacentor variabilis (Say). J. Med. Entomol. 19 (6):734-742.

10. Dees, W.H., D.E. Sonenshine, and E. Breidling. Ecdysteroids in Hyalomma dromedarii and Dermacentor variabilis and their effect on sex pheromone activity. Paper presented at VIth Internatl. Congr. Acarology, September 5-12, 1982, Edinburgh, Scotland. Ellis Harwood, Publishers (in press).

11. Khalil, G.M., D.E. Sonenshine, R. Milton Silverstein, P.J. Homsher, and H. Hoogstrall. Sex pheromones of Ixodidae. IBID (in press).

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13. Sonenshine, D.E. "Tick Pheromones." Chapter in "Current Topics in Pathogen-Victor-Host Relationships." K.F. Harris (ed) Praeger Scientific, NY, NY (in press, accepted April 9, 1983).

14. Jaffe, H., D.E. Sonenshine, M. Thompson, and E. Breidling. Controlled release reservoir system for the delivery of insect steroid hormone analogues against ticks. Internal. Symposium on Controlled Release of Bioactive Materials. July 24-27, 1983, San Francisco, CA, Manuscripts in preparation.

15. Shepherd, J., J.H. Oliver, Jr., J.D. Hall. A polypeptide from male accessory glands which triggers maturation of tick spermatozoa. Inter. J. Invert. Rep. 5: 129-137, 1982.

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17. Pound, J.M. and J.H. Oliver, Jr. Synganglial and Neurosecretory Morphology of Female Ornithodoros parkeri (Cooley) (Acari:Argasidae). J. Morphology 173: 159-177, 1982.

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